# Enantiomeric Profiling of Chiral Biomarkers

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# *Enantiomeric Profiling of Chiral Biomarkers*

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**Abstract**

Wastewater-based epidemiology (WBE) is an innovative tool that, unlike traditional epidemiological approaches, is capable of providing real-time profiling of community health and lifestyle along with emerging trends and changes in pattern usage of drugs in wastewater. By using human urinary excreted indicators, so-called "biomarkers", WBE provides estimates at population level. Therefore, the choice and the evaluation of suitable biomarkers of exposure to drugs is of fundamental importance for the public health monitoring. Moreover, since many drugs are chiral, the investigation on enantiomeric profiling of chiral biomarkers provides a new dimension to WBE. To aid enantiomeric profiling in WBE, sensitive enantioselective methodologies are required.

In this thesis, two novel multiresidue analytical methods based on chiral liquid chromatography coupled to mass spectrometry were developed and validated.

The first method investigated the main human biomarkers for the detection of illicit drugs of abuse and potentially abused licit drugs. New biomarkers were investigated, such as mephedrone, PMA and all MDMA's metabolites. Furthermore, a case study on mephedrone posed the basis for a novel approach towards biomarker selection in estimation of human exposure to chiral drugs with limited metabolism data. As a result, mephedrone was a suitable biomarker due to its stability in wastewater. In addition, some of its metabolites were also proposed as potential candidates for mephedrone use.

The second method explored biomarkers of quinolones' use, as they represent one class of antibiotics with rising concern in antibiotic resistance. The most comprehensive panel of quinolones' biomarkers was considered for the first time in WBE studies.

Both methodologies were applied to wastewaters from eight locations in Europe allowing the first pan-European studies on enantiomeric profiling of chiral biomarkers. Key findings of this research included: the detection of high mephedrone loads only in the UK, thus indicating human consumption; the detection of HMMA, a MDMA metabolite, as a suitable indicator of MDMA consumption and the determination of different synthetic production routes of methamphetamine across Europe. With regards to quinolones' biomarkers, higher ofloxacin loads were found in Southern European cities along with differences in

enantiomeric fraction with respect to Northern ones. Moreover, ofloxacin's metabolites showed ofloxacin use and ulifloxacin was found as a result of prulifloxacin consumption.

Therefore, enantiomeric profiling led to an understanding of: (i) new patterns of emerging drugs of abuse, (ii) changes in patterns of classical illicit drugs and (iii) quinolones with the verification of the origin of drug residue, potency of abused drug and its synthetic route and (iv) quinolones' metabolic profiles.

Moreover, the simultaneous determination of quinolones' biomarkers in European samples allowed for the verification of spatial and temporal trends of quinolones' use and the occurrence of their resistance genes. This proof-of-concept research will facilitate further advances in the WBE field.

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**List of abbreviations**

| | |
|---|---|
| ACN | Acetonitrile |
| AEME | Anhydroecgonine methyl ester |
| AFP | α-Fetoprotein |
| AGC | Automatic gain control |
| AGP | $\alpha_1$-acid glycoprotein |
| AMP | Amphetamine |
| AMR | Antimicrobial resistance |
| ARG | Antibiotic resistance gene |
| BE | Benzoylecgonine |
| BOD | Biochemical Oxygen Demand |
| BSA | Bovin serum albumin |
| BZP | Benzylpiperazine |
| CBH | Cellobiohydrolase |
| CAS | Chemical Abstracts Service |
| CD | Circular dichroism |
| CE | Collision energy |
| CEA | Carcinoembryonic antigen |
| CF | Correction factors |
| CLED | Cysteine-, lactose- and electrolyte-deficient |
| CNS | Central Nervous System |
| COC | Cocaine |
| COD | Chemical Oxygen Demand |
| CSPs | Chiral stationary phases |
| CV | Cone voltage |
| DDA | Data dependent acquisition |
| DDD | Defined daily dose |
| ddMS2 | Data dependent MS/MS |
| DF | Diastereomeric fraction |
| DHMA | 3,4-dihydroxymethamphetamine |
| DMDCS | Dimethyldichlorosilane |
| DMV | Desmethylvenlafaxine |
| DTR | Drug target residue |

| | |
|---|---|
| CG | Chroriogonadotropin |
| EARS-Net | European Antimicrobial Resistance Surveillance Network |
| ECDC | European Centre for Disease Prevention and Control |
| EDDP | 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine |
| EMCDDA | European Monitoring Centre for Drugs and Drug Addiction |
| EF | Enantiomeric fraction |
| EFSA | European Food Safety Authority |
| EMA | European Medicines Agency |
| ESAC-Net | European Surveillance of Antimicrobial Consumption Network |
| ESI | Electrospray ionisation |
| ESPAD | European School Survey Project on Alcohol and Other Drugs |
| EtOH | Ethanol |
| EWS | Early Warning System |
| Exp | Experimental |
| GC-MS | Gas chromatography coupled to mass spectrometry |
| HD | High-Definition |
| H-ESI | Heated electrospray ionisation |
| HLB | Hydrophilic-lipophilic balanced |
| HMA | 4-hydroxy-3-methoxyamphetamine |
| HMMA | 4-hydroxy-3-methoxy-methamphetamine |
| HPLC | High performance liquid chromatography |
| HPLC-HRMS | High performance liquid chromatography coupled with high resolution mass spectrometry |
| HPLC-MS/MS | High performance liquid chromatography coupled with tandem mass spectrometry |
| HRMS | High resolution mass spectrometry |
| HAS | Human serum albumin |
| IDL | Instrumental limit of detection |
| ILIS | Isotopically-labelled internal standards |
| IPA | Isopropanol |
| IQL | Instrumental limit of quantification |

| | |
|---|---|
| IS | Internal standard |
| IT | Injection time |
| ITN | Initial Training Network |
| LC | Liquid chromatography |
| LC-MS/MS | Liquid chromatography coupled with tandem mass spectrometry |
| LC-MS | Liquid chromatography coupled with mass spectrometry |
| LC-VP | Liquid chromatography-Velos Pro |
| LC QTOF | Liquid chromatography coupled with quadrupole time-of-flight |
| LC TQD | Liquid chromatography coupled with triple quadrupole |
| LLE | Liquid-liquid extraction |
| LOQ | Limit of quantification |
| MAX | Mixed-mode anion exchange |
| m-CPP | *meta*-chlorophenylpiperazine |
| MCX | Mixed-mode cation exchange |
| MDA | 3,4-methylenedioxyamphetamine |
| MDL | Method detection limit |
| MDMA | 3,4-methylenedioxy-methamphetamine |
| MDEA | 3,4-methylenedioxy-*N*-ethyl-amphetamine |
| ME | Matrix effect |
| MeOH | Methanol |
| METH | Methamphetamine |
| MP | Mobile phase |
| MQL | Method quantification limit |
| MRM | Multiple Reaction Monitoring |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry |
| MW | Molecular weight |
| 6-MAM | 6-monoacetylmorphine |
| N | Nitrogen |
| NPS | New psychoactive substances |
| NSAIDs | Non-steroidal anti-inflammatory drugs |

| | |
|---|---|
| OS | Other sources |
| OVM | Ovomucoid |
| P | Phosphorus |
| PDE5 | phosphodiesterase-5 |
| pI | Isoelectric point |
| pHLM | pooled human liver microsomes |
| PMA | *para*-methoxy-amphetamine |
| PMMA | *para*-methoxy-methylamphetamine |
| Pred | Predicted |
| PTFE | Polytetrafluoroethylene |
| RRT | Relative retention time |
| Rs | Resolution |
| RSD | Relative standard deviation |
| SCORE | Sewage Analysis CORe group Europe |
| S/N | Signal-to-noise |
| SOD | Superoxide Dismutase |
| SPE | Solid-phase extraction |
| THC | Tetrahydrocannabinol |
| TFMPP | Trifluoro-methylphenylpiperazine |
| UNODC | United Nations Office on Drugs and Crime |
| QqQ | Triple quadrupole |
| VEN | Venlafaxine |
| WBE | Wastewater-based epidemiology |
| WHO | World Health Organization |
| WW | Wastewater |
| WWTP | Wastewater treatment plant |

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# Chapter 1: Introduction

## 1.1 Wastewater Analysis for Human Health Biomarkers Detection

Determinants of health-related states are generally investigated through epidemiologic methodologies, such as population surveys and surveillance studies, which allow for a comprehensive evaluation of community-wide status. However, these methods are associated with a significant time lag, so that there is a need for real-time profiling assessment of community health and lifestyle, especially in the case of outbreaks and hot points. For this reason, a lack of real-time data has been the *causa movens* for a new monitoring program capable of guaranteeing an integrated approach to the current monitoring techniques. Such a program consists of real-time measurements of human health indicators through wastewater analysis.

Testing wastewater for obtaining information at community level is a pioneering approach. Although still in its initial stages, it is currently used as a complementary tool in the determination of illicit drug use trends at community level through the analysis of human urinary biomarkers in wastewater.

In this context the research project SEWPROF, funded by the European Commission, Marie Curie Actions, Seventh Framework Programme, Initial Training Network (ITN), aimed at developing inter-disciplinary and cross-sectorial research capabilities in the newly-emerging field of wastewater-based epidemiology (WBE).

## 1.2 The SEWPROF project

SEWPROF project provided an integrated approach towards public health monitoring based on innovative WBE techniques. It involved 14 researchers working on 12 different countries and it operated on four research streams, which contributed to depict the Europe-wide monitoring of societal health and lifestyle through WBE. These addressed new developments on:

1. *Robust sampling techniques and assessment of biomarker transformation in sewers*. Areas of investigation were focused on: passive samplers for wastewater biomarkers, adaptation of traditional sampling devices for WBE and new models for estimating in-sewer transformation of biomarkers.
2. *Methods for targeted analysis and screening of biomarkers*. Research was dedicated to provide novel methods for the detection of biomarkers by using hyphenated analytical and bioanalytical techniques. In particular, specific health-related biomarkers in wastewater were targeted for their analysis by chromatography coupled with mass spectrometry. These included new drugs/psychoactive compounds and their transformation products (TPs), illicit drugs, $F_2$-isoprostane, urinary metabolites of pesticides, antibiotic residues, phosphodiesterase type 5 inhibitors and their metabolites. By using enantioselective analysis, other health-related biomarkers were chiral illicit drugs, chiral potentially abused drugs and chiral antibiotics. Cocaine and prostate specific antigen were targeted by employing newly developed multichannel biosensors.
3. *Estimation of community wide-health and lifestyle.* It provided real-time monitoring of the selected biomarkers along with identification of new illicit drug trends, estimates of population habits and comparison with social studies.
4. *Europe-wide wastewater epidemiology monitoring.* It provided spatial and temporal variations of the above-mentioned biomarkers across several European locations.

Interlinked research was carried out through secondments and visits among the institutions involved along with an international training program on main WBE topics.

## 1.3 PhD aims and objectives

This PhD was part of the second macro-area of research within SEWPROF project. It aimed at developing and validating new analytical methodologies capable of determining the enantiomeric profiling of chiral biomarkers in wastewater as an aid to understand human exposure to chiral xenobiotic substances. It also contributed to the fourth area as novel developed methods were applied to the Europe-wide environmental monitoring.

The objectives of this PhD were the following:

- To develop and validate a robust, multi-residue and sensitive methodology for the analysis of chiral biomarkers for illicit drug use at enantiomeric level in wastewater. This was because the analysis of human biomarkers could give invaluable information regarding the pathways of human exposure due to stereoselective metabolism of some chiral illicit drugs.
- To investigate enantiomeric profiling in estimation of illicit and potentially abused drug use via WBE for the (i) verification of their potency, origin and route of administration; (ii) monitoring of changing patterns of their use; (iii) legal and illicit use distinction and (iv) identification of metabolic pathways of drugs of abuse in wastewater.
- To develop and validate a chiral analytical method for the determination of biomarkers for chiral antibiotics' use.
- To explore the enantiomeric profiling of chiral antibiotics (i.e. quinolones) in wastewater by applying the WBE approach.
- To perform target and untargeted screening of unknowns, metabolites and transformation by-products at enantiomeric level.

Finally, my PhD contributed to the identification of biomarkers of exposure to antibiotics in wastewater and to their analysis along with the biomarkers of microbial resistance.

## 1.4 Secondments

In order to accomplish the above mentioned objectives of this PhD, as established by the SEWPROF project, a number of secondments at European academic institutions was carried out. These are as follows:

- *Secondment at the Universitat des Saarlandes (USAAR), Dept. of "Experimentelle und Klinische pharmakologie und Toxikologie" (Homburg, Germany).* In order to investigate the enantiomeric profiling of an emerging drug of abuse, mephedrone, a number of experiments was performed to support the study on biomarkers of mephedrone intake. These were aimed at (i) studying differences in enantiomeric fractions of mephedrone and normephedrone in biological matrices (i.e. rat urine and pooled human liver microsomes) and (ii) looking at the presence of other mephedrone metabolites in incubated wastewater through high resolution mass spectrometry (HRMS).
- *Secondment at "Istituto di Ricerche Farmacologiche Mario Negri" (IRCCS) (Milan, Italy).* A preliminary investigation on biomarkers of exposure to antibiotics was performed through a method in HPLC-MS (QqQ). These were 24 antibiotics belonging to several classes, such as quinolones, fluoroquinolones, cephalosporins, macrolides, carbapenems, sulphamidics, tetracyclines, broad-spectrum penicillins, urinary-tract infections and glycopeptides.
- *Secondment at "Institut Catala' de Recerca de l'Aigua" (ICRA) (Gerona, Spain).* In-sewer stability and transformation study on pharmaceuticals and illicit drugs in a pressurized sewer under anaerobic conditions was performed. This enabled (i) the targeted screening of compounds in wastewater through the usage of advanced analytical methodologies, based on UPLC-Q-Trap analysis; (ii) the screening of suspected compounds in wastewater through UHPLC-HR-MS/MS analysis for investigating the presence of nitro transformation products of selected compounds and (iii) the study of in-sewer stability of illicit drugs present in the samples.

## 1.5 Thesis Outline

The first chapter presents an introduction about the needs for real-time monitoring of human health biomarkers through wastewater analysis and the

contribution of the SEWPROF project to enhance the knowledge on WBE field through the investigation areas. It also reports the aims and objectives of this PhD and the topics of the secondments planned in the European institutions.

The second chapter shows an overview on human health biomarkers and methodologies used for testing wastewater from the scientific literature, highlighting their benefits and current limitations. WBE approaches in the context of multi-scale communities are reported along with a discussion on the contribution from enantiomeric analysis.

The methodologies and results sections are presented and discussed in two main parts:

- Part A. *Biomarkers for illicit drugs' use.*

The third chapter is dedicated to a novel multiresidue method developed for the simultaneous detection and the quantification of 56 achiral and chiral biomarkers in wastewater. These are illicit drugs and potentially abused drugs. Method development phase is reported in details. The methodology was applied to a week monitoring campaign in the UK. Results on in-sewer stability and transformation study in a pressurized sewer under anaerobic conditions were also discussed.

After finding a new emergent drug from the monitoring campaign at consistent levels in the wastewater, chapter four poses the basis for a new approach towards biomarker selection in estimation of human exposure to chiral drugs with limited metabolism data. Particularly, a case study on mephedrone is discussed here.

Chapter five shows the results from the first enantiomeric profiling of illicit drugs in a pan-European study, giving a massive contribute to the Europe-wide WBE monitoring.

- Part B. *Biomarkers for antibiotics' use.*

The sixth chapter explores a new multi-residue stereoisomeric analysis of human and veterinary chiral quinolones drugs (including an antifungal drug) in wastewater using chiral liquid chromatography coupled with tandem mass spectrometry. Method development is largely discussed.

Chapter seven covers the first study on enantiomeric profiling of quinolones and monitoring in European wastewaters along with a first attempt to correlate WBE to the occurrence of quinolones resistance gene.

Finally, the conclusions of this thesis are reported in chapter eight. In particular, a summary of the results, highlights and novel contributions achieved in the previous chapters are shown. This thesis concludes with an outlook on future research activities and directions based on the outcomes of this PhD.

Appendixes are provided as supplementary materials to the thesis. For clarity reasons, tables and figures are reported under their corresponding chapters throughout text.

# Chapter 2: Background and Literature Review

## 2.1 Monitoring and Evaluation of Human Health State

Health information is the result of several multi-integrated monitoring indicators and their measurement strategies. Its comprehensive assessment for the public health decision making is generally provided by the World Health Organization (WHO) through analytical and descriptive studies, surveillance and statistics on health themes [1]. This institution is the directing and coordinating authority for health within the United Nations System [2]. Health data are published regularly in annual reports and they are available online. The drawback of this assessment is represented by the delay in information flows from the population to the policies' makers, which can affect the progress and performance of strategies. Therefore, real time profiling of community-wide health and lifestyle is necessary for the investigation of determinants of health-related states.

Among many approaches, an innovative one is the *wastewater analysis*. The novelty is represented by the possibility to evaluate population health through the study of biomarkers. In this manner, a newly-emerging field of wastewater-based epidemiology (WBE) has been developed, with the purpose of estimating population habits (e.g. consumption or exposure to xenobiotics). The research strategy for obtaining epidemiological information from wastewater was initially applied only to a restricted class of compounds such as drugs of abuse, in order to determine community-wide illicit drug use trends. This is accomplished via the analysis of urinary drug biomarkers in wastewater with the usage of analytical methodologies, including hyphenated mass spectrometry (MS) techniques. This original approach towards WBE profiling area, once extended to other health biomarkers, will be able to take an instantaneous picture of a community by describing aspects and behaviours in real-time, through a wider range of techniques including bioanalytical tools and real-time sensors.

### 2.1.1 European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)

The European centre for the monitoring of drugs and drug addiction provides a surveillance activity through several networks cooperating together. These include reitox focal points, key epidemiological indicators, Early Warning System (EWS) network and legal correspondents [3]. In addition, new developments, such as supply indicators and European School Survey Project on Alcohol and Other Drugs (ESPAD), are also involved in order to draw the complex picture of drug use. Typical key epidemiological indicators are listed below:

- treatment demand, which consists of the number of people that require a therapeutic treatment after drug use;
- general population surveys;
- problem drug use;
- drug related deaths, coming from hospitals' reports;
- infectious diseases.

Along with these more traditional tools, new complementary monitoring approaches are expanding their reliability in giving information on drugs use: trend spotter methods, internet surveys and wastewater monitoring. Especially the latter

tool has seen a growing interest in the scientific public. In occasion of the conference "Testing the waters", held in Lisbon in May 2013, the state-of-the-art of this emerging field was presented through a multidisciplinary and international approach. In the report "EMCDDA insights-Assessing illicit drugs in wastewater" [4], an overview on community drug use information achievable through wastewater analysis was presented. The most recent developments of wastewater analysis were shown at the 2nd international conference "Testing the Waters 2015" last October and published in "Assessing illicit drugs in wastewater: advances in wastewater-based drug epidemiology" by EMCDDA last March as a demonstration of how WBE field is rapidly growing.

## 2.2 Human Health Biomarkers: Definition

Biomarkers in health field are defined "(…) as indicators of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (Biomarkers Definition Working Group, (2001) [5]). There are two major types of biomarkers: biomarkers of exposure, which are used in risk prediction, and biomarkers of disease, which are used in screening, diagnosis and monitoring of disease progression [6]. The application of the biomarkers concept is mainly associated to the clinical field. However, as humans are subjected to a range of chemical exposures from the environment, it is possible to define biomarkers of exposure as indicators of daily life exposure to chemicals in air, water, soil, food and lifestyle factors [7]. Exposure to chemicals can be either voluntary such as food, pharmaceuticals and illicit drugs, or involuntary such as personal care products absorbed through the body, pollutants and contaminants present in the air and in the food, inhaled or ingested. Biomarkers of exposure can also be useful indicators about lifestyle and human habits. For instance, they can be indicators of intake of alcohol, consumption of food, but also chemicals such as prescribed pharmaceuticals [8, 9]. In particular, the rationale behind the assessment of biomarkers of exposure is that chemicals once in the body undergo absorption, distribution, metabolism and excretion, as in the case of pharmaceuticals, before they are found in the sewage system.

## 2.3 Wastewater Analysis

As stated by the EMCDDA's report [4], a "wastewater system contains information about the society that serves and reflects the behaviours of that population". As highlighted by Bohannon (2007) [10], a wastewater treatment plant (WWTP) has many advantages. In fact, it can be intended as an "accessible, economical source of real-time and pooled epidemiologic information" [10].

Moreover, the research on drugs in wastewater analysis can be intended as the means to provide so-called "drug consumption index", that is more appropriate for drug surveillance purposes [4]. Indeed, it can provide a "screening tool for communities" served by the WWTP monitored.

Daughton in his work [11] showed that if an excretion product remains constant in wastewater and during the transport to the WWTP, the quantity excreted by a community is roughly correspondent to that found in the wastewater system. In this way, the criteria for the assessment of a biomarker were posed. Examples of suggested and existing biomarkers in wastewater are reported in Table 2-1.

The step from the wastewater analysis to a more comprehensive and systematic approach able to assess the collective exposure of the population of a community to any sort of chemicals was called *wastewater-based epidemiology*.

**Table 2-1** Potential and existing wastewater biomarkers of human health (modified from Thomas and Reid (2011) [12]).

| **Health parameter** | | **Biomarkers** |
|---|---|---|
| *Lifestyle* | Alcohol consumption | Ethyl sulphate |
| | Tobacco consumption | Cotinine, tobacco specific nitrosamines, menthol |
| | Drug consumption | Drugs and their urinary metabolites (i.e. cocaine, benzoylecgonine, ecgonine methyl ester, tetrahydrocannabinol (THC), 11-nor-9-carboxy-THC, synthetic cannabinoids, amphetamine, methamphetamine, cathine, cathinone, MDMA, 6-monoacetylmorphine (6-MAM), together with the new and emerging synthetic drug classes, etc.) |
| | Anabolic steroid abuse | Synthetic steroids and their metabolites |
| *Diet* | | Urinary sugars |
| | | Synthetic sweeteners |
| | | Phytoestrogens |
| | Fruit and vegetable intake | Flavonoids |

| | | |
|---|---|---|
| | Soya | Iso-flavonoids |
| | Caffeine consumption | Caffeine |
| | Meat consumption | Creatinine, taurine, 1-methylhistidine, 3-methylhistidine |
| *Health* | Illness and disease | Specific pharmaceuticals and their metabolites |
| | Oxidative stress | $F_2$-isoprostanes, 8-hydroxydeoxyguanosine |
| | Pregnancy | Human chorionic gonadotropin |
| | Allergy | Antihistamines |
| *Cancer* | Hepatoma, testicular, colon, breast, pancreatic | α-Fetoprotein (AFP, cancer), carcinoembryonic antigen (CEA) |
| | Prostate | PSA |
| | Ovarian | CA12.5 |
| | Breast | CA15.3 |
| | Gastrointestinal | CA19.9 |
| | B cell dyscrasias | Immunoglobulin |
| | Testicular cancer, trophoblastic tumors | Chroriogonadotropin (hCG) |
| Environment | PAH exposure | Phenanthrol and other PAH metabolites |
| | Aflatoxin exposure | Aflatoxin N(7)-guanine |

### 2.3.1 Critical Steps in Wastewater Analysis: Sample Preparation

Wastewater is a very complex and dynamic environmental matrix. It contains lots of contaminants able to compete and interfere with the investigated target compounds. So, "cleaning" the wastewater sample with an adequate sample preparation is crucial. The following aspects need to be considered:

- Pre-treatment of the collected sample and/or adoption of precautions in order to not degrade the analytes of interests.
- Choice of sample volume, which often is a compromise between large volumes (able to permit good recoveries and low limits of detection and quantification) and small volumes (representing a good starting point for the pre-concentration step, but more negatively influenced by the matrix effect).
- Removal of solid part of the wastewater sample by one or more filtration steps.
- Pre-concentration of the analytes through mainly solid phase extraction (SPE) in order to get concentration range of microgram/liter (µg/L) or nanogram/liter (ng/L).

A good compromise in the number of the steps in the sample preparation is also required. Indeed, many steps may cause either the partial loss of the analyte with a consequent decrease in recovery or the complete absence.

One of the first papers, which highlighted the discrepancies in misreporting data of wastewater analysis, was written by Baker et al. (2011) [13]. They drew attention to critical steps during the analytical methodologies used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs. As mentioned, they pointed out the importance of: (i) the effect of evaporation temperature and solvent with regards to the SPE extracts; (ii) the effect of silanising glassware; (iii) the recovery of analytes during vacuum filtration through glass fibre filters and the pre LC–MS filter membranes and (iv) stability data of analytes. Van Nuijs et al. (2012) [14] emphasised that in most cases degradation of the analytes occurred after few hours the collection. One of the most updated review on stability of abused drug biomarkers was recently published by McCall et al. (2015) [15].

### 2.3.2 Critical Steps in Wastewater Epidemiology: Uncertainties

Understanding the main uncertainties related to WBE approach is crucial in the wastewater analysis. The lack of biodegradation data on biomarkers is still an area of further investigation. Stability is linked to the fate of a drug from the excretion step to the wastewater flow, but other parameters are involved in a fate of a drug. Degradation, partitioning and sorption of drug target residues (DTRs) are also important variables to consider [16], [17].

Among these sources of uncertainty, special attention was drawn to the estimation of the population size served by a WWTP as an essential component for comparing results from different sites. Ort et al. (2010) [18] showed how to maximise the sampling procedure in order to avoid evaluation errors associated to the catchment size, sewer type, sampling setup, substance of interest and accuracy of analytical method. The authors emphasised that sampling, chemical analysis, flow measurement, excretion fraction and estimated number of people that contributed to the wastewater are components of uncertainty. Lai et al. (2011) [19] illustrated that differences or changes in drug loads lower than 20-30% of uncertainty were not significant. This enabled to define when real changes in mass loads occurred.

## 2.4 Wastewater-based Epidemiology

WBE is a new methodology for estimating the mass load of human biomarkers (detected in wastewater) in a population [20]. Its concept, originally developed by Zuccato et al. (2005) [21], consists of determining the levels of illicit drugs and their metabolites in wastewater and back-calculating the mass loads of the parent drugs associated with the population under investigation. Once the drug metabolisms and the excretion patterns are known, these loads can then be used to evaluate the consumption of drugs in g $day^{-1}$ or doses $day^{-1}$. A schematic overview of the WBE approach is reported in Figure 2-1.

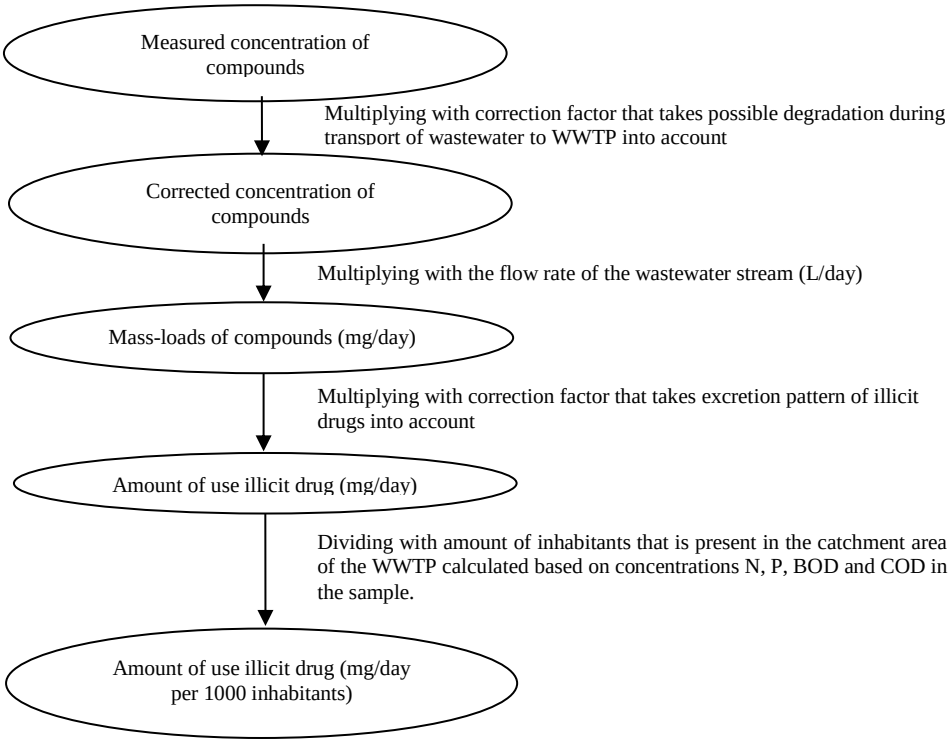


**Figure 2-1** Overview of the WBE approach (modified from Van Nuijs et al.[22]) (N: nitrogen, P: phosphorus, BOD: Biochemical Oxygen Demand, COD: Chemical Oxygen Demand).

As highlighted by Zuccato et al. (2008) [16], the first step of a WBE approach is the selection of a major and exclusive excretion product, named drug target residue (DTR), that ideally needs to satisfy the criteria of stability in wastewater. The DTRs are urinary metabolites or unchanged parent compounds (see Table 2-2). For some compounds, the metabolites are more reliable DTRs, as in the case of benzoylecgonine, the major cocaine metabolite, whilst for others the parent compound works better, as in the case of methamphetamine. During the metabolism, many compounds are excreted as glucuronides, but these conjugates are very unstable in wastewater [23] as they are deconjugated by faecal bacteria

enzymes to the free compound [24]. For this reason, glucuronides are not suitable DTRs.

**Table 2-2** Examples of DTRs selected for a study in WBE (modified from Zuccato et al. [16]).

| Drug | DTR | Relation of DTR to parent drug | Percentage of drug dose excreted as DTR[a] | Molar mass ratio (parent drug/DTR) | Correction factor |
|---|---|---|---|---|---|
| Cocaine | Benzoylecgonine | Major metabolite | 45 | 1.05 | 2.33 |
| | Cocaine | Parent drug (minor excretion product) | | | |
| Heroin | Morphine | Major but nonexclusive metabolite | 42 | 1.29 | 3.07 |
| | 6-MAM | Minor but nonexclusive metabolite | | | |
| Amphetamine | Amphetamine | Parent drug and major excretion product | 30 | 1.0 | 3.3 |
| Methamphetamine | Methamphetamine | Parent drug and major excretion product | 43 | 1.0 | 2.3 |
| MDMA | MDMA | Parent drug and major excretion product | 65 | 1.0 | 1.5 |
| Cannabis | THC-COOH | Major metabolite of THC (cannabis active principle) | 0.6 | 0.91 | 152 |

[a] Average for the most frequent route of intake.

As reported in Baker et al. (2012) [25], the daily load is determined by the following factors:

- DTR's concentration (*Conc*) present in wastewater, expressed in ng $L^{-1}$.
- The stability change, intended as a percentage of the change of each compound in raw wastewater at a certain temperature and after an estimated time (*Stab*). In Baker and Kasprzyk-Hordern, (2011) [26], this value was positive in the case of an increase in DTRs' concentration or negative in the case of a decrease.
- DTR's sorption to suspended particulate matter (SPM) (*Sorp*), expressed as a concentration in ng $L^{-1}$. As highlighted in Baker et al. (2012) [25], this parameter was considered in order not to underestimate the concentration. In literature, data on the sorption are still missing for many compounds.

- The flow of an influent wastewater system, calculated as a volume of wastewater during 24 hours in L day$^{-1}$ (*Flow*).

The daily load, expressed in Eq. (1) as g day$^{-1}$, is a suitable starting point for the comparison of occurrence of drugs in a wastewater system.

$$g\ day^{-1} = \frac{\left\{\left[Conc.\times\left(\frac{Stab}{100-Stab}+1\right)+Sorp\right]\times Flow\right\}}{1\times 10^{9}} \quad (1)$$

The step from daily load to daily consumption requires other parameters. Indeed, in order to determine the daily consumption, the information on the population of a wastewater catchment area is needed. Moreover, other parameters linked to DTR are involved along with the load data. These are:

- the percentage of dose excreted as DTR after relevant forms of administration (*Excretion*);
- the molar ratio between the parent drug and its DTR ($MW_{Ratio}$);
- the DTR present as contribution from other sources (*OS*), different from that one of the parent compound (expressed in mg day$^{-1}$ 1000 people $^{-1}$).

The equation for the daily estimation, calculated in mg day$^{-1}$ 1000 people$^{-1}$, is as follows:

$$mg\ day^{-1}\,1000\ \ people^{-1} = \frac{\left[(Load\ \times 1000)\times\left(\frac{100}{Excretion}\right)\times MW_{Ratio}\right]}{\frac{Population}{1000}} - OS \quad (2)$$

Through the described approach, it is possible to achieve an evidence-based and real-time estimation of drug collective consumption.

### 2.4.1 Human Indicators

Human urine indicators play a very important role in drug estimation, because they could be potentially used as index mass loads [26]. The population of a certain area may vary not only during a year due to immigration flows, but also it may fluctuate during the day for work reasons. Also weather conditions, e.g. heavy rains or dryness, play a relevant role in determining the volume of wastewater. Therefore, all these variables could affect the estimation of the population size and, consequently, the back-calculation of drugs use. Along with classical human indicators of the wastewater analysis, such as BOD, COD, phosphorus, ammonia

[27], other substances were considered. Coprostanol was targeted by Daughton [28]. Lai et al. (2011) [19] found acesulfame suitable for estimating population, even though the lack of national consumption data was determinant for its exclusion. Creatinine was the elected human indicator by Chiaia et al.[29], whilst cotinine and 1,7-dimethylxanthine were retained suitable by Baker [26]. Caffeine was often considered together with nicotine. The latter compound was also widely used as a biomarker for environmental tobacco smoke (ETS) in order to identify people at risk and verify the progress of tobacco control strategies resulting in less ETS. Recently, a multiple substances panel approach able to give a proper assessment of the population was preferred. This was because this approach was (i) more robust compared to the singular-substance-based, (ii) statistically framework orientated and (iii) more advantageous for larger wastewater catchments.

Chen et al. (2014) [30] suggested that drug consumption per capita could be calculated without an estimation of the population by using a population biomarker (PB). The five criteria established for a good PB were defined as follows:

1. quantification level in wastewater at μg $L^{-1}$ range;
2. low affinity for SPM or to filter paper;
3. stability in wastewater matrix;
4. constant excretion;
5. exclusive human excretion in order to have a better correlation with census population data.

Equation (3) was used for estimating drug consumption per capita:

$$Drug\ \ consumptio\ \ n\ per\ \ capita\ = \left( \frac{DTR\ \ concentrat\ \ ion}{PB\ \ concentrat\ \ ion} \right) \times \left( \frac{PB\ \ excretion\ \ \ rate}{DTR\ \ excretion\ \ \ factor} \right) \quad (3)$$

Spatial and temporal differences on occurrence of drug consumption can be studied at every community levels. Indeed, its evaluation can be achieved at local, national and international level.

### 2.4.2 Local level

At local level, many studies were independently performed in several countries. As an example, from a study in 2008 in Milan (Italy) [16], it was possible to see variations in DTRs from the most investigated illicit drugs through wastewater analysis in a two-week time (Figure 2-2). From the obtained profile, it

was observed that THC-COOH (metabolite of THC, which is one of the major components of cannabis) remained stable as morphine during all the week, ecstasy showed a slight increase and cocaine experienced substantial increase during the weekend.

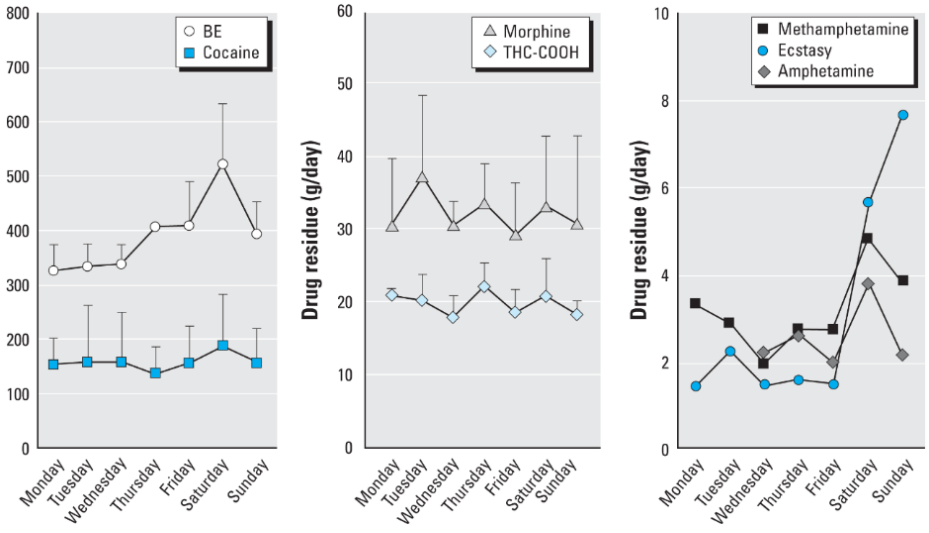


**Figure 2-2** Drug residue loads for the city of Milan (Italy) in 2008 (from Zuccato et al. 2008 [16], reproduced with permission from Environmental Health Perspectives).

Bones et al. (2007) showed an opposite pattern for cocaine and its metabolite (higher for cocaine than for benzoylecgonine) in Dublin (Ireland) [31]. This was the only discordant pattern with respect to the results found in other studies as stated by Van Nuijs et al. (2011) [32].

Weekly variations were studied in Zagreb (Croatia) by Terzic et al. (2010) [33], showing an increased heroin drug profile with respect to those observed in western European countries and an overall DTRs' increase during weekend days. Similar studies were performed in Paris (France) by Karolak et al. (2010) [34] and in three Canadian cities by Metcalfe et al. (2010) [35].

A case study by Postigo et al. (2011) illustrated fluctuations in drugs consumption in a range time of a week and in few months monitoring in a prison (Figure 2-3) [36]. The drug consumption in this particular condition was lower than the estimated intake in a nearby city. Methadone, alprazolam and ephedrine were the most consumed drugs. A decrease of usage of methadone and alprazolam was noted on Sunday, presumably due to the absence of the prisoners for the weekend permits and an increase of ephedrine in correspondence of more cases of asthma and bronchitis.

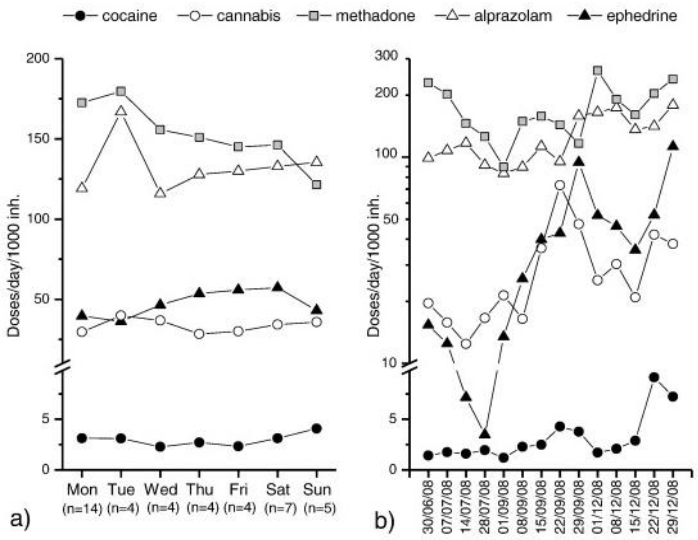


**Figure 2-3** Estimates for cocaine, cannabis, methadone, alprazolam and ephedrine consumption: a) use throughout the week and b) use during the studied period, both expressed in doses/day/1000 inhabitants (reproduced with permission from Fig. 1 in Postigo et al. (2011) [36], Elsevier).

In order to show patterns of drug usage not only in a week time but also in a day time, a study was successfully performed in Oslo (Norway) by Reid et al. [37]. The day scale investigation showed how pharmaceuticals and illicit drugs patterns changed under their kinetics of drug-flow profiles (Figure 2-4).

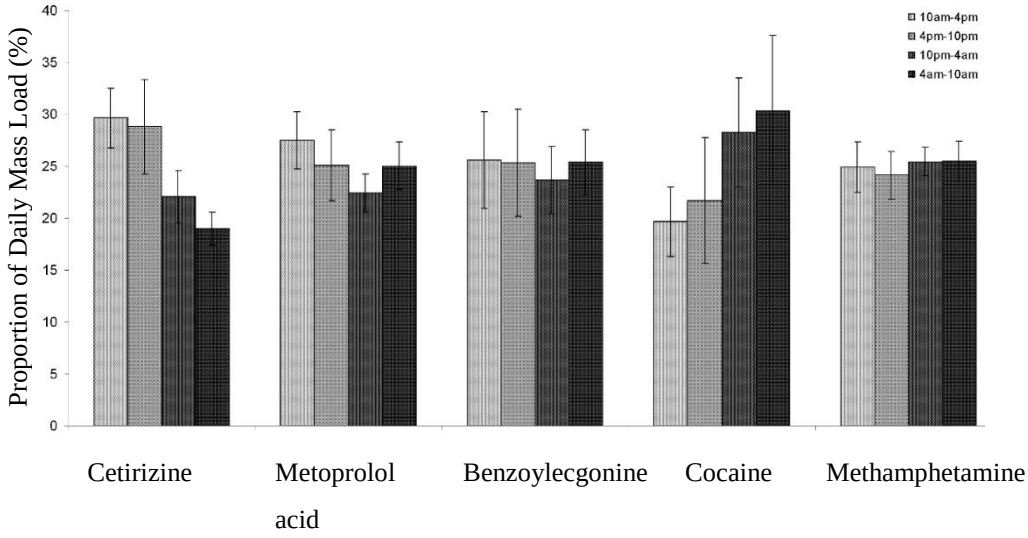


**Figure 2-4** Proportion (mean, *n* = 26) of the daily mass-load of cocaine (excreted unchanged), benzoylecgonine, methamphetamine, cetirizine and metoprolol acid that are excreted at different times of the day. Data derived from 6-hourly measurements of sewage in a treatment plant (after the primary settling tank) in Oslo throughout the period of 4–30 September 2009. Times are therefore off-set by 7 h to account for the average residence time of sewage in the pipe-network (reproduced with permission from Fig. 4 in Reid et al. (2011) [37], Elsevier).

In particular, it was pointed out that a diurnal trend was present for cetirizine, but not for metoprolol acid due to its pharmacokinetics, different doses and the possible *nocturia* once ingested. The stable tendency of methamphetamine was explained because of complex combination factors between the half-life excretion and the different dosing times. Interesting considerations arose from the cocaine. It was mostly consumed during the evening hours as the unchanged excreted cocaine showed very high percentages in the proportion of the mass load, whilst its metabolite, which has a long excretion half-life, was constant during the day and the night. Through the comparison of the cocaine patterns in weekdays and weekend days it was possible to observe that its consumption in the weekend was later in the evening with respect to weekdays. In fact, the profile of the excreted unchanged cocaine culminated between 10 pm and 4 am in weekdays and, later, between 4 am and 10 am during the weekend (Figure 2-5).

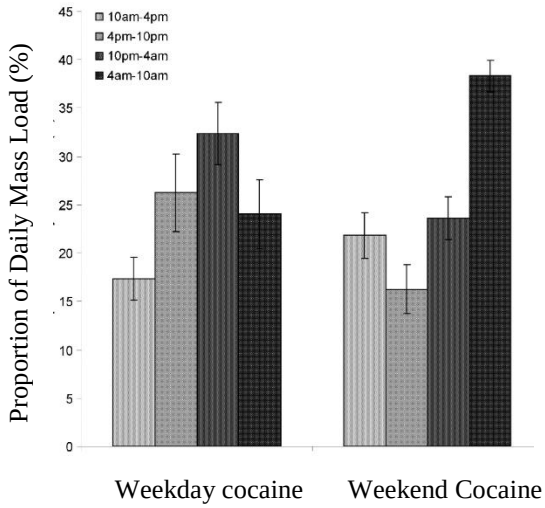


**Figure 2-5** Proportion of the daily mass-load of cocaine (excreted unchanged) with comparison between weekday (Monday–Friday) and weekend (Saturday and Sunday) periods. Data derived from 6-hourly measurements of sewage in a treatment plant (after the primary settling tank) in Oslo throughout the period of 4–30 September 2009. Times are therefore off-set by 7 h to account for the average residence time of sewage in the pipe-network (reproduced with permission from Fig. 5 in Reid et al. (2011) [37], Elsevier).

Year timescale temporal variations enabled critical considerations on illicit drugs' use trends. Zuccato et al. (2011) [38] showed these differences through wastewater analysis. The study proved a significant drop in cocaine and heroin consumption in 2009 with a decrease of 45% and 66% respectively. These results were also

confirmed by the weekly load profile. The cannabis pattern, instead, seemed to be slightly decreased from the beginning of 2005 until March 2009, but an increase was observed in September 2009 at level similar to that one seen in 2005. The methamphetamine pattern revealed a constant rise from 2005 to 2009. Several hypotheses were postulated in order to explain the observed drop in cocaine and heroin trends. Along with the decrease of number of consumers and the increase of cheaper illicit drugs and interest for NPSs, the economic crisis surely played an important role in the cocaine and heroin trends. Wastewater analysis data were also confirmed by local and general epidemiological surveys, even though these documents were published only few years later in 2011. This case by Zuccato is the evidence that WBE can reveal the estimation of illicit drug trends faster than traditional methods.

### 2.4.3 National level

At national level, one of the first inter-cities studies was performed by Zuccato et al. (2005) [21], in which samples were collected from four medium-sized Italian cities. In that study, loads measured for cocaine's DTR were similar in Cuneo, Latina and Varese (average 33±3 g $day^{-1}$), with exception for the largest city from Sardinia island, Cagliari (130 g $day^{-1}$). Although the assessment of cocaine load did not include Southern Italian cities, this inter-cities comparison was a first attempt in WBE field. With the inclusion of the city of Milan in a later study [16], spatial variations were studied about cocaine use in Italy: this illicit drug seemed to be used more in the big city with respect to the medium-sized cities as stated by Van Nuijs et al. (2011) [39]. The confirmation of this finding arrived from a further study conducted in the "Aqua Drugs project", in which five large-sized, four medium-sized, four medium-small-sized and four small-sized Italian cities were compared at the same time. Moreover, in this study a more critical evaluation of cocaine consumption through the North, the Centre and the South of Italy was performed. It showed that the highest cocaine use was in the central part of the country, whilst comparable lower trends were observed in the North and in the South.

Van Nuijs et al. [40] collected data from a one-year campaign in Belgium. Variations in the number of inhabitants were taken into account for the estimation of drug consumption [22]. As before, it was confirmed that cocaine was more

consumed in large cities, such as Antwerp, Brussels and Charleroi, than in medium-sized cities. Seasonality of cocaine consumption was also investigated in Belgium [41]. Van Nuijs did not note any statistically significant variations of cocaine usage along the seasons. This result was in contrast with findings obtained by Huerta-Fontela et al. (2008) in surface water from North-East of Spain cities [42]. Van Nuijs contested the results obtained from the Spanish study due to a lack of statistics and for the chosen matrix. Temporal trends were studied by Postigo et al. (2010) [43] in many cities in Ebro river basin area in a year time. Spatial variations were investigated also in urban and rural areas in Oregon (USA) by Banta-Green (2009) [44]. Urban areas showed higher benzoylecgonine loads than rural ones, whilst for methamphetamine the pattern was quite widespread in all the municipalities.

Spatial and temporal variations were also studied in urban, semi-rural and vacation areas in Australia by Lai et al. (2013) [45]. A further example of spatial differences at national level was carried out in France by Nefau et al. (2013) [46], where geographical significant differences were found due to inhomogeneous drug pattern in the country.

WBE was also applied to investigate illicit drug trends in megacities. Khan et al. (2014) [47] studied this approach for the first time in four Chinese megacities (Beijing, Guangzhou, Shenzhen, and Shanghai), populated by 11.4 million of inhabitants for two-month period (September-October) in 2012. Considered the restricted surveillance data in this area of the world, this study showed consistency with the United Nations Office on Drugs and Crime (UNODC) report data. In fact, higher consumptions in cocaine, ecstasy, methamphetamine and ketamine were observed in the southern megacities of Guangzhou and Shenzhen with respect to those detected in the northern Beijing and Shanghai. The following patterns were reported: methamphetamine was ubiquitous; ketamine was mostly used in the South of China; NPSs' levels were low, presumably indicating a scarce interest for these substances, except for benzylpiperazine, meta-chlorophenylpiperazine (mCPP) and trifluoro-methylphenylpiperazine (TFMPP) probably used as substitutes of MDMA. Cocaine and MDMA were found in lower amount compared to Europe. In contrast with Thibault (2012) [48], who stated that the elective drug of abuse for "marginalized rural Chinese or migrant workers living on the outskirts of urban areas" was heroin, in this study heroin metabolite's loads in megacities was not

statistically relevant [47]. Due to the lack of seasonal prevalence data, WBE approach should be more investigated in these areas in future studies.

### 2.4.4 International level

At European level, one of the first approaches in using WBE was realised by Castiglioni et al. in 2006, in which data obtained from two WWTPs in two European countries, Italy and Switzerland, were compared [23].

Later, Zuccato et al. (2008) enhanced this process by including data from three big cities, Milan (Italy), Lugano (Switzerland) and London (UK) [16]. From Zuccato's work, it was possible to confirm that the trend in illicit drugs use was THC >> cocaine > heroin with the exception of Milan, where cocaine use was higher than cannabis. The most evident discrepancy observed among these cities was ascribed to amphetamine loads which were statistically significant only for London. Regarding to ecstasy, slightly higher consumption was detected in Lugano than in Milan and London. The measurements performed by wastewater analysis were in accordance with UNODC report 2006 at national scale [49].

In 2011, Van Nuijs reviewed the most important studies overall Europe performed by several research groups [32], emphasising how the need of a procedural standardisation should be pursued in order to use WBE for routine drug monitoring. The intent of more coordinated studies through an European-wide network (Sewage analysis CORE group- SCORE) became tangible with the creation of a "common protocol of action". It also placed the basis for the success of the 19 cities study and reinforced a subsequent wider program including 27 cities. In detail, a common methodology with a standardisation of procedures of wastewater, named "the best practise protocol", was given by Castiglioni et al. (2014) in order to get more homogeneous data for the comparison among countries (Table 2-3) [50].

The European study by Thomas et al. 2012 [51], involving nineteen cities across the continent, highlighted regional differences in drug usage (Figure 2-6). In order to get valid quality assurance data for the analytical methodologies used, an inter-laboratories test was performed, for the first time, before the one-week campaign. If quality control results failed the attended concentrations for a drug, the results obtained for that substance within the monitoring were not included for that laboratory.

**Table 2-3** Summary of the consensus best practice protocol for the sampling, analysis and reporting of data elaborated by the SCORE group (modified from Castiglioni et al. 2014 [50]).

| Parameter | Agreed procedure | Further comment |
|---|---|---|
| *Sampling and sampling handling* | | |
| Sampling information | A questionnaire for each sewer network should be completed | The questionnaire elaborated by Ort is available in Castiglioni et al. (2013) |
| Sampling point | 1st routine influent sampling location at works | To be noted in sampling questionnaire |
| Sample type | 24 h composite | |
| Defined day | Start/finish between 8 and 10 am | |
| Sampling container | PET or glass(silanised) | Record |
| Storage treatment during sampling | <4 ºC | Record time and temperature in storage |
| Storage after sampling | Choose based on the available options in the following preferential order:<br>1.On SPE cartridge within 12 h with internal standard added.<br>2.Freeze preferentially after addition of internal standard.<br>3.Freeze | Record period before extraction.<br>Time in freezer if frozen. |
| Filtration | Internal standard added before filtration.<br>Filter type GFC (0.45 µm) | Record any deviation. |
| Additional parameters to be recorded in sampling questionnaire | BOD, COD, N, P, flow data, type of sewage influent, temperature, pH | Report methods also if possible |
| *Compounds to be analysed* | Cocaine, benzoylecgonine, amphetamine, methamphetamine, MDMA, THC-COOH | All participants are welcome to include other compounds (e.g. heroin, 6-MAM, morphine, mephedrone, ketamine, GHB) |
| *Quality control* | | |
| Internal quality control | Use of labelled internal standards for each analyte | Report any deviation |
| External quality control | Analysis of methanol standards and influent samples prepared by one laboratory and sent to each participant | Interlaboratory studies |
| *Data reporting* | Common procedure to calculate limits of detection and quantification<br>Samples results as a means of three individual analyses | Reporting template is provided |

Questionnaires were filled providing information about wastewater catchment area and details on sample preparation. The sampling campaign took place simultaneously for all the laboratories with a sampling time between 8 am and 10 am. One week in March was chosen, with the exception of only those cities hosting particular events or festivals in that week. Composite 24-hours samples were collected, stored as according to protocol's specifications and analysed with internal

validated analytical methods. A key step before the analysis was the use of internal standard in order to reduce and compensate the errors linked to the matrix effect and to the analytical technique. The procedural standardisation of the study guaranteed a harmonization of the collected data and enabled comparison of daily drug loads, showing interesting trends for cocaine, amphetamine, methamphetamine, ecstasy and cannabis across Europe.

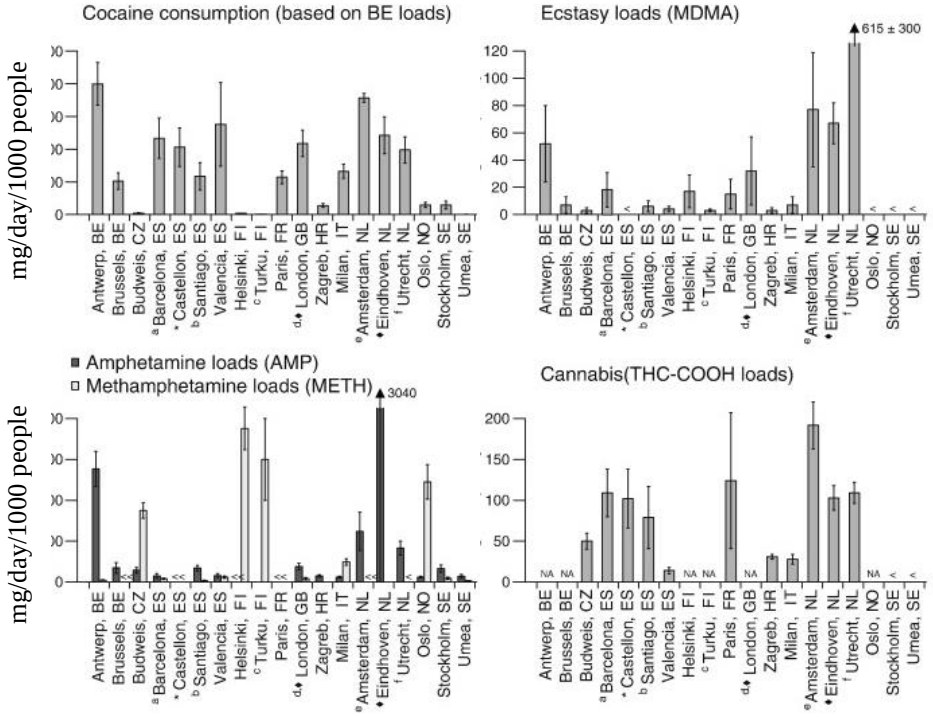


**Figure 2-6** Average estimates of cocaine consumption (back-calculated from benzoylecgonine BE loads) and population-normalized loads of amphetamine (AMP), methamphetamine (METH) in 19 selected European cities and cannabis (THC-COOH, all in mg/1000 inhabitants/day) in 13 of them between the 9th and 15th March 2011 (mean ± SD from all sampling days, n = 7) (reproduced with permission from Fig. 2 in Thomas et al. (2012) [51], Elsevier).

Unlike widespread cannabis trends observed in all Europe with no substantial differences, geographical variances were noticed. Western and central European areas revealed more cocaine consumption than eastern and northern ones. Ecstasy was more prevalent in the UK and in Dutch areas, especially due to the presence of illicit production sites. Northern Europe showed more consume of methamphetamine. For countries with more cities included in the study, such as Sweden and Finland, cocaine loads were higher in more urbanised cities than in less urbanised ones. Temporal dissimilarities were noticed in cocaine and MDMA consumptions: the ecstasy pattern revealed the recreational use of the drug

especially due to its change in weekend loads. Indeed, this was more evident in ecstasy than in cocaine (Figure 2-7).

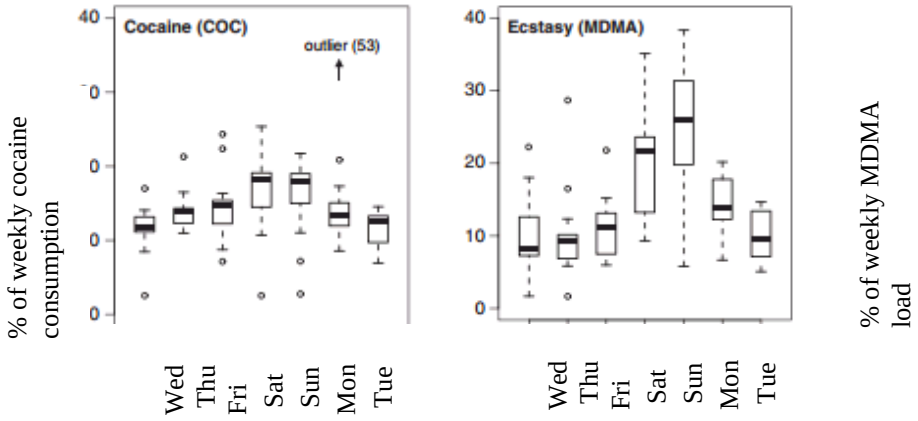


**Figure 2-7** Day-to-day variation of cocaine consumption (based on the loads of the main metabolite benzoylecgonine) in 19 cities and ecstasy (MDMA) in 15 cities. Medians are significantly different on the weekend compared to weekdays (reproduced with permission from Fig. 3 in Thomas et al. (2012) [51], Elsevier).

Despite of general agreement of the results with national prevalence data, an increased number of cities involved was highly recommended for further monitoring campaigns.

In fact, from 14.12 million of people and 19 cities involved in the first monitoring campaign in 2011, 11.50 million and 23 cities were in 2012, up to 24.74 million and 42 cities in 2013. Results were displayed as in the example shown in Figure 2-8 [52]. According to that study, in the case of cocaine, WBE data were not in fully agreement with the national reports (i.e. constant trend instead of a decrease). By including data from Germany, it was possible to better describe the cocaine use across Europe. Indeed, cocaine was more highly consumed in western Europe than in eastern part. The methamphetamine usage was more an eastern-northern phenomenon. Cannabis remained used very widespread, with the exception of Amsterdam due to a non-resident population consumption.

Inability in estimating large doses used by few people or vice versa and differences linked to the drug purity still remain limitations of wastewater analysis. Several suggestions were provided to support data interpretation in future monitoring campaigns:

- demographic data should be taken into account as cities have different demographic profiles and wastewater-based data cannot be compared in terms of

drug consumption if high and low prevalence areas are used. Indeed, it would be preferable to assess for each country a panel of cities with differences in demographics that allows for comparison at international level;

- monitoring strategies should be improved in terms of logistics, quality assurance and representativeness for the whole year.

An improved systematic WBE approach, including integrated small, medium and large-sized cities profile and more monitoring weeks during the year (i.e. seasonal drug monitoring) would result in a better contribute for reliable data on the assessment of drug consumption. This achievement was realised, for the first time, at continental level.

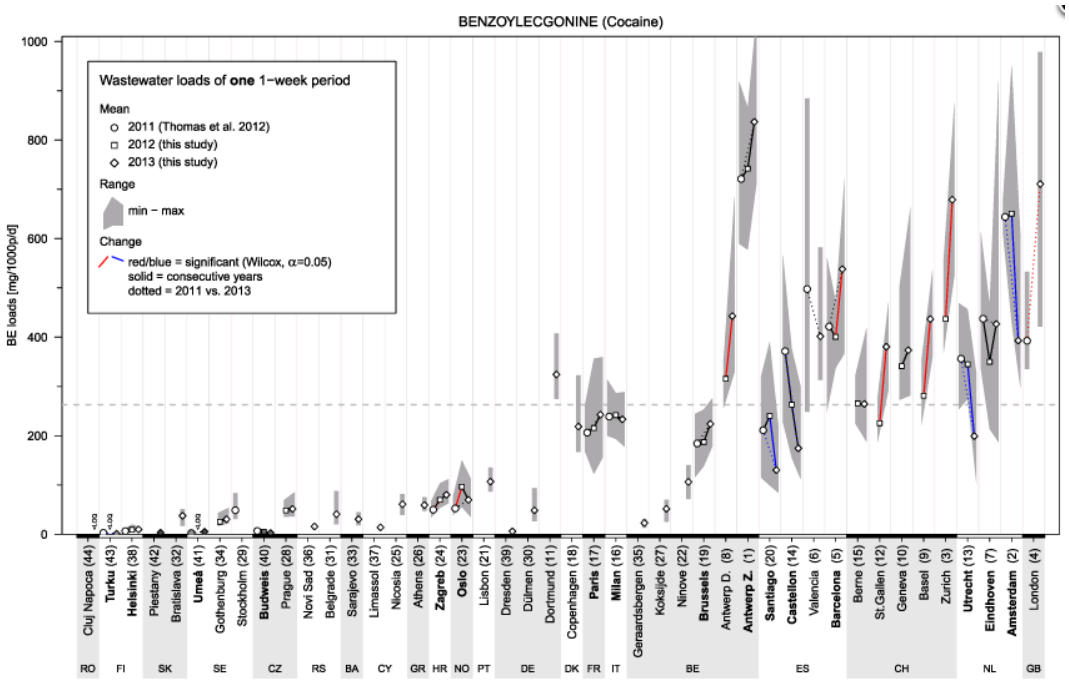


**Figure 2-8** Population-normalized benzoylecgonine (BE) loads of a single 1-week period per year (extracted from Ort et al. (2014) [52]).

A similar approach is still missing in other areas of the world, where knowledge in this field is fragmentary. A detailed geographical and spatial situation is still unknown for the poorest area of the world. An estimation of illicit drugs trends in some areas of the world was provided only by Khan et al. (2014) [47]. Briefly, cocaine, MDMA and amphetamine consumption was higher in America, Canada, Australia and Europe than in the mainland China, even if a recent decrease was observed in Australia for MDMA [53]. Methamphetamine use was more prevalent in America than in Asia and lower in Europe, with the exception of some countries,

such as Finland, Norway and Czech Replublic. Ketamine consumption was lower in Europe (not including the UK), and in America compared to Asia. Cannabis also showed the same trend of cocaine.

## 2.5 Enantiomeric analysis

### 2.5.1 Chirality

As human pharmacokinetics shows stereoselectivity in the case of many chiral xenobiotics, chirality is a relevant topic to investigate.

According to the IUPAC Recommendations 1997, chirality is defined as "the geometric property of a rigid object (or spatial arrangement of points or atoms) of being non-superimposable on its mirror image". Enantiomers are identical in most of their chemical-physical properties, except for the ability in rotating the plane-polarized light. This feature reflects also a different way of interaction between the molecule and the biological receptor due to a particular spatial conformation of the ligands. In nature, the selector often is a biological molecule with a specific conformation able to give not only weak interactions, such as hydrogen bonds and dipole interactions, but also steric effects, which can create inclusion or protrusion pockets suitable for the molecule fitting. As a consequence, potency and activity of enantiomers are highly influenced. In fact, among non-steroidal anti-inflammatory drugs (NSAIDs) *S*-ibuprofen is one hundred times more potent than *R*-enantiomer, whilst among beta-blockers *S*-enantiomers are highly active in humans [54].

In the past, drugs were usually administered as racemate due to the high costs of enantioselective syntheses for pharmaceutical companies. Only recently, an inversion of the trend has been observed as clinical trials costs are doubled in the case of a racemate compared to those for a single enantiomer.

Different behaviours of enantiomers need to be evaluated during the development process of a drug.

Differences related to the distribution of a drug in tissues may occur. An example is given by venlafaxine, which is a drug administered in racemic form. In rats a different distribution of *R*- and *S*- enantiomer in the serum and in the brain was observed [55].

Metabolism studies are extremely important. Historically, the under-investigation of *in vivo* metabolism of thalidomide was known for its dramatic consequences in foetus [54]. This drug has the tranquilising properties in its *R*-enantiomer and a

dangerous teratogenic activity in *S*-enantiomer [54]. If administered to women in gestation, thalidomide interconverts in the body to the hazardous *S*-enantiomer.

Not all the chiral drugs undergo an *in vivo* interconversion, but many other phenomena may also occur [54]:

- differences in metabolic rate can facilitate the production of a compound with a determined stereochemistry with respect to its enantiomer;
- differences in the metabolic pathway can favour a particular enantiomer;
- changes in the number of chiral centres, such as introduction of another chiral centre (i.e. the product of the *R*-warfarin, called warfarin alcohol, has two chiral centres with respect to the original drug) or removal of the chiral centre (i.e. achiral sulfone originated from chiral omeprazole) or conservation of the chiral centre (i.e. the hydroxylated metabolites of warfarin, produced by both enantiomers, maintain the same chiral centre of warfarin);
- enzymatic chiral inversion;
- different stereoselectivity among species (i.e. the clearance of propranolol is higher for the *S*-enantiomer in dogs than in humans).

Metabolites, coming from a chiral compound, may have only one pharmacologically active enantiomer or both active enantiomers with the same potency or different activities [54].

### 2.5.2 Environmental analysis: chiral chromatography coupled with tandem mass spectrometry using protein-based chiral stationary phases

Chiral liquid chromatography (LC) environmental analysis mainly uses macromolecular selectors as stationary phases [56]. The interest on these protein-based chiral stationary phases (CSPs) arose because chiral distinction capability of enzymes and plasma proteins was known as natural chiral pool of selectors [57]. Those based on albumins (e.g. human and bovine serum albumins, HSA and BSA), glycoproteins [e.g. α1-acid glycoprotein (AGP) and crude ovomucoid (OVM)], enzymes, such as cellobiohydrolase I (CBH) belong to this group [57]. Enantiomer separation capabilities cover preferably acidic compounds in the case of HSA column, basic ones in the case of CBH and a wide range for AGP.

Since many illicit drugs are basic, the most used for environmental chiral analysis of drugs of abuse are CBH and AGP columns.

#### 2.5.2.1 CBH and AGP columns

CBH has a cellobiohydrolase immobilized on spherical 5 μm silica particles as chiral selector. Its isoelectric point (pI), defined as the pH at which a substance is electrically neutral, is 3.9. According to Henriksson et al. [58], three main active chiral-recognition areas are defined in the column: a catalytically active core, a connecting area and a cellobiohydrolase domain with 36 aminoacids forming two disulphide-bridged loops. The catalytically active core contains the dominating chiral binding site, whilst the cellulose one has the other enantioselective site [59].

Developed by Hermansson [60], AGP consists of a single peptide chain with 181 aminoacids and five heteropolysaccaride units, containing 14 residues of sialic acid. Due to the presence of this acidic component, AGP has 2.7 as pI [61]. Sugar moieties are also present [60]. As the tertiary structure is missing, few information are available on the chiral recognition sites and mechanism of AGP, even if it is known that hydrophobic, electrostatic and hydrogen bonding interaction play a key role in the retention and enantioselectivity of a solute in AGP [62].

#### 2.5.2.2 Chiral separation and mechanism

Factors, such as temperature, pH and mobile phase composition, could influence the chiral recognition. Changes in temperature were very limited especially for CBH because its chiral selectors could be denaturated [63]. Hence, pH could be a key parameter along with mobile phase compositions. Both columns are positively charged at pH<pI and negatively charged at pH>pI. This means that from pH>pI up to neutral conditions, the degree of the net negative charge of the chiral selector increases, thus determining ionic bonds between the chiral CSP and the positively charged solute (i.e. amine). As a consequence, a high retention time (Rt) along with enantioselectivity are expected. Hydrophobic interaction and hydrogen bonds are also forces involved in Rt and their influence depends on the nature of the solute. The enantioselective retention could be achieved through mobile phases containing different organic modifiers, different aqueous-organic ratios and different salt concentrations. The most frequently used uncharged organic modifiers are methanol, acetonitrile, 2-propanol. Depending on their nature, a decreasing modifier concentration will result in a higher Rt and enantioselectivity (i.e. amines). The presence of a buffer in a mobile phase can ionise the analytes and alter the interactions of the analytes with the stationary phase at molecular level.

#### 2.5.2.3 Sample preparation needs and constraints

In order to study the enantiomeric profiling of illicit drugs in the environment, some precautions are required during the sample collection and preparation. Indeed, an incorrect assessment of the relative concentration of enantiomers could be obtained at this stage due to the activation of enantioselective and/or enantiospecific microbial processes [13]. Potentially abused substances such as antidepressants were found not stable. In particular, nortriptyline, venlafaxine, fluoxetine and norfluoxetine reached half concentration after 24 h at pH 7.4 [13]. In that case, time and pH were two factors responsible for the degradation process (i.e stability was improved at lower pH). Moreover, enantioselective degradation occurred when influent and effluent samples were compared [64, 65]. In order to reduce enantioselective degradation, a correct storage protocol is highly recommended. The time existing between the sampling and the sample preparation needs to be reduced. Samples kept at low temperatures during the transport will help in minimizing the microbial activity.

A controversial question is about the acidification of the sample and the addition of sodium azide as the matrix might be subjected to modification [56]. If the advantage of both processes relies on stopping the microbial activity, additional errors could be inevitably introduced (i.e. at lower pH the percentage of sorption to solids components in wastewater can vary considerably for ionisable compounds). In MS the most often used source for chiral analysis of drugs of abuse is the electrospray ionisation (ESI). Signal suppression may occur in ESI, thus significantly affecting the matrix effect and the chiral recognition for the enantiomers [56]. Indeed, extreme pH cannot be used due to possible denaturation of the chiral selector itself. As a consequence, a key step in chiral method development is the choice of the SPE sorbent. The elective choice is represented by Oasis hydrophilic-lipophilic balanced (HLB) cartridges. In fact, for amphetamines an Oasis mixed-mode anion exchange sorbent (MAX) and HLB were preferred to an Oasis mixed-mode cation exchange one (MCX) with a CBH column. This was due to the eluting agent influencing the decrease of enantiomeric resolution [65].

### 2.5.3 Enantiomeric profiling of illicit drugs in environmental analysis

The investigation of illicit drugs at enantiomeric level in environmental samples was principally performed (i) to remove pharmaceutically active

compounds and (ii) to understand the difference between the direct consumption and its disposal, as well as the origin of a drug residue in WBE. An indication of which enantiomer fraction is predominant is given by the measurement of enantiomeric fraction (EF). EF is the ratio between the peak area of the first eluted enantiomer (or the (+)-enantiomer, if (-)-enantiomer is used in the numerator, it is usually indicated in the equation) and the sum of both enantiomer peak areas (EF= (+)/[(+)+(-)]). A chiral CBH column was used to perform environmental chiral analysis of amphetamine-like compounds [66].

Kasprzyk-Hordern et al. (2010) [65] showed how the enantiomeric composition of chiral drugs was altered during the wastewater treatment process, thus determining an occurrence of enantioselective processes during wastewater treatment. Indeed, venlafaxine was found racemic in influent and enriched of one enantiomer in effluent wastewater samples in two WWTPs.

The hypothesis about the enantioselective degradation of drugs in the WWTP process was confirmed in a later study conducted in seven WWTPs in England over the period of nine months in 2012 [67]. In raw wastewater, *R*-(-)-MDMA was found predominant with respect to the *S*-(+)-MDMA due to stereoselective human metabolism. EF value increased from 0.68 in raw wastewater to 0.78 in treated wastewater due to the treatment of wastewater possibly resulted from the stereoselective microbial processes.

Enantiomer-specific degradation was observed for amphetamine, leading to an enrichment of *R*-(-)-form. In the case of ephedrine, the natural *1R,2S*-(-)-enantiomer was detected in raw wastewater, whilst the synthetic *1S,2R*-(+)-enantiomer was found in treated wastewater, thus showing perhaps a chiral inversion process. Receiving waters were also enriched of *R*-(-)-enantiomer for amphetamine (EFs > 0.80) and MDMA (EF up to 0.80±0.01) and *1S,2R*-(+)-ephedrine.

Microcosm experiment carried out with a CBH column under isocratic conditions proved that stereoselective processes leaded to an enrichment of *R*-(-)-amphetamine (EF changed from 0.47 to <0.02) [68]. The enantiomeric fate of MDA changed along the treatment of wastewater. Indeed, EF equals to 0.28-0.30 was reported in raw wastewater indicating a prevalence of *S*-(+)-form, a slight enrichment of *R*-(-)-MDA during wastewater treatment (EF=0.38-0.40) and finally EF=0.56-0.58 in surface waters, thus leading to the *R*-(-)-enantiomer due to stereoselective removal or transformation of *S*-form [67]. For a potentially abused antidepressant,

venlafaxine, removal is problematic in WWTP treatment due to weak stereoselective processes. Low venlafaxine degradation was further proved by Vazquez-Roig et al. [69].

### 2.5.4 Enantiomeric profiling of illicit drugs for WBE purposes

Only few papers correlated the enantiomeric composition of illicit drugs found in wastewater to official statistics.

Chiral analysis helped in distinguishing the MDMA origin in wastewater and clarifying whether it was due to direct disposal or its abuse. All illicit synthesis methods produce racemic MDMA (EF=0.5). However, *S*-(+)-MDMA undergoes preferential metabolism over *R*-(-)-MDMA. This leads to the enrichment of residual MDMA excreted in urine (and then found in wastewater) with the *R*-(-)-enantiomer (EF>0.5). This characteristic change in EF of MDMA allows for the verification of whether drug residues present in wastewater results from its consumption (EF>0.5) or direct disposal of unused drug (EF=0.5). For example, the results of several sampling campaigns in England (Figure 2-9) [70] and in the Netherlands [71] showed that MDMA is usually present in wastewater due to its consumption (EF>0.5; MDMA enriched with *R*-(-)-enantiomer). However, excessively high mass loads of MDMA during one sampling campaign in a WWTP in Utrecht (the Netherlands) proved to be racemic, thus indicating direct disposal of unused MDMA. This coincided with a police raid earlier at a nearby illegal production facility within the catchment (Figure 2-10) [71].

The study of the enantiomeric profiling of MDA can also help in recognising the origin of a drug residue. The minor metabolic pathway of MDMA produces MDA with an enrichment of *S*-(+)-enantiomer in urine. Differently, if the MDA is originated from its direct disposal in the racemic form, the *R*-(-)-enantiomer would be prevalent due to the metabolic conversion from *S*-(+)-MDA to *R*-(-)-MDA [72]. Indeed, it was observed by Kasprzyk-Hordern et al. [70] that MDA was detected only in August with an enrichment of its *S*-(+)-enantiomer. The presence of MDA in raw wastewater with EF equals to 0.24 and 0.30 seemed to be due to MDMA abuse and not to its intentional consumption. No intentional MDA use was also found in Dutch cities.

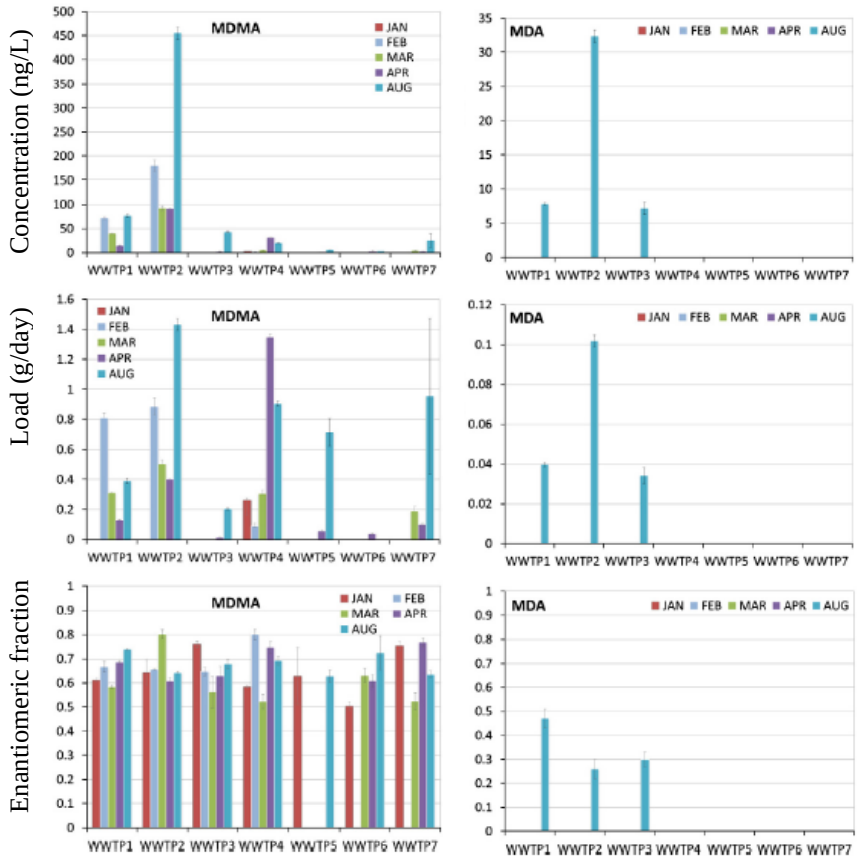


**Figure 2-9** Concentrations, loads and enantiomeric fractions found in wastewater for MDMA (left) and MDA (right) in the UK (reproduced with permission from Fig. 3-4 in Kasprzyk-Hordern and Baker (2012) [70], Elsevier).

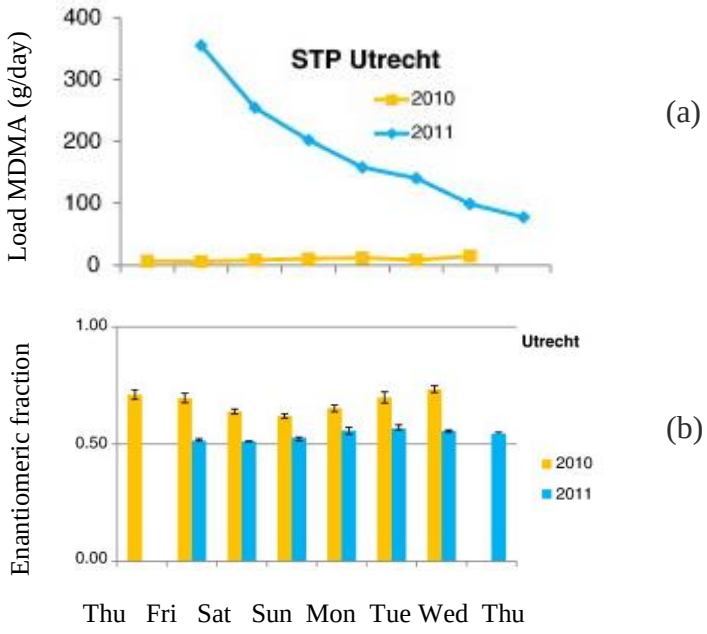


**Figure 2-10** (a) Loads of MDMA of wastewater samples in Utrecht collected for the European Monitoring campaign in 2010 and 2011, (b) EF of MDMA in Utrecht for those campaigns (modified from Emke et al. [71]).

For some drugs, such as amphetamine, the contribution from other drugs' metabolism needs to be considerated. Kasprzyk and Baker [67] found that legal amphetamine, prescribed in *S*-(+)-form in England, was in low levels when compared to the illegal use. EF average was 0.64 in raw watewater, indicating an enrichment of *R*-(-)-enantiomer. These data were in agreement with human consumption as *S*-(+)-amphetamine is preferentially metabolised after ingestion of the racemate. In a monitoring campaign in 2011 in Eindhoven (the Netherlands), high amphetamine loads were found 14 times higher than those in 2010 (Figure 2-11). EF values were 0.52±0.01 and 0.52±0.02 in Eindhoven for the campaign in 2010 and 2011, respectively. This data did not match with the EFs found in England. The enantiomeric profiling of amphetamine and methamphetamine is still not well assessed due to discrepancies on data coming from different countries.

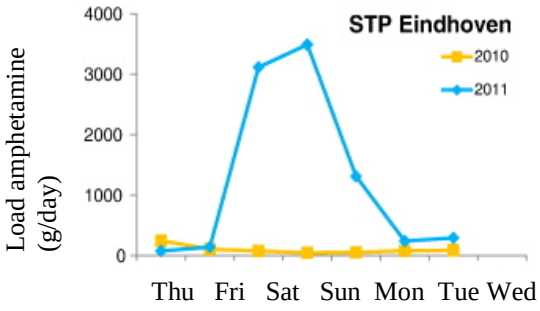


**Figure 2-11** Daily load of amphetamine in Eindhoven for the European Monitoring campaign in 2010 and 2011 (from Emke et al. 2014 [71]).

Usage patterns of chiral drug usage was also studied in Valencia region by Vazquez-Roig [69].

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# Chapter 3: Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry

## 3.1 Summary

This chapter proposes a novel multi-residue stereoselective method utilising a CBH (cellobiohydrolase) column for the analysis of 56 drug biomarkers in wastewater. These are: opioid analgesics, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, phosphodiesterase-5 (PDE5) inhibitors, amphetamine and methamphetamine drug precursors. Satisfactory enantiomeric separation was obtained for 18 pairs of enantiomers including amphetamine, methamphetamine, MDMA (3,4-methylenedioxy-methamphetamine) and its metabolites HMA (4-hydroxy-3-methoxyamphetamine) and HMMA (4-hydroxy-3-methoxy-methamphetamine), PMA (*para*-

methoxyamphetamine), MDA ((±)-3,4-methylenedioxyamphetamine) and mephedrone. The method was applied in a one week monitoring study of a large wastewater treatment plant in England, UK. Most target drugs were found at quantifiable concentrations in analysed samples. Enantiomeric profiling revealed that amphetamine, methamphetamine and MDMA were found enriched with *R*-(-)-enantiomers, probably due to their stereoselective metabolism favouring *S*-(+)-enantiomers. MDA was either enriched with *R*-(-)- or *S*-(+)-enantiomer indicating that its presence might be due to either abuse of racemic MDA or abuse of MDMA respectively. Non-racemic enantiomeric fractions were also observed in the case of HMMA and mephedrone suggesting enantioselective metabolism. To the author's knowledge, this is the first time chiral separation and wastewater profiling of mephedrone, PMA, MDMA and its metabolites HMA and HMMA have been reported.

## 3.2 Introduction

Wastewater-based epidemiology (WBE) has the potential to inform public health via the analysis of human urinary biomarkers in wastewater [1]. WBE is an emerging field but it has already found applications in verifying spatial and temporal community-wide illicit drug [2, 3], alcohol [4] or tobacco use [5].

An understanding of human pharmacokinetics and the selection of potential biomarkers informing public health is key to successful application of the WBE approach. As human pharmacokinetics shows stereoselectivity in the case of many chiral xenobiotics [6], chirality is also important to investigate in WBE.

In a recent study, the enantioselective separation of common illicit drugs revealed changes in enantiomeric composition of chiral drugs during wastewater treatment [7, 8]. In particular, it was demonstrated that the type of chiral drug, the treatment technology used in a wastewater treatment plant and the season affected the stereoselective enrichment or depletion of the enantiomeric composition of a drug. In another study, microbial metabolic processes were found to be responsible for stereoselective degradation of amphetamine-like compounds in river [9] and activated sludge microcosms [10].

Unfortunately in WBE, chiral analysis still has limited application despite its high potential in helping to understand for example: (i) the different route of synthesis of the drugs, (ii) the differentiation between the abuse and the licit use of drugs, (iii)

the origin of a drug residue, differentiation between consumption and disposal of unused drugs and (iv) and the potency of the abused drug [11]. The concept of enantiomeric profiling in WBE has been applied for the first time by Kasprzyk-Hordern et al. (2012) [11]. In fact, from a study conducted in 7 WWTPs in England for 5 months, it was possible to conclude that MDMA was found in the influent wastewater samples due to its abuse rather than its direct disposal. Also, the presence of MDA was associated with abuse of MDMA and not abuse of MDA. In another study by Emke et al. (2014) [12], chiral analysis was key in confirming that unexpectedly high loads of MDMA observed in wastewater from one of Dutch cities were a result of dumping of MDMA from a local production facility during a police raid.

In order to undertake enantiomeric profiling of wastewater for chiral drug biomarkers, robust and multi-residue chiral analytical methods need to be developed. Until now, chiral LC-MS (liquid chromatography coupled with tandem mass spectrometry) methods were utilised in the investigation of a limited number of chiral drugs [11-13]. Therefore, chiral methods were used only as complementary tools alongside non-chiral LC-MS methods. Those approaches required an ad hoc sample preparation, which meant higher sample volume, more time consuming and less cost effective analysis.

This chapter proposes, for the first time, a multi-residue method utilising a CBH (cellobiohydrolase) column for the analysis of 56 drug biomarkers at enantiomeric level, including satisfactory enantiomeric separations for 18 pairs of enantiomers. To the author's knowledge, this method is the first to allow for:

(i) simultaneous and mutiresidue differentiation between the abuse and the licit use of drugs (e.g. in the case of amphetamine as illicit amphetamine, as opposed to prescribed licit amphetamine, is distributed as racemate),

(ii) verification of the origin of a drug residue (e.g. methamphetamine as chiral signature of methamphetamine is route of synthesis dependent),

(iii) differentiation between consumption and disposal of unused drugs (e.g. in the case of MDMA, fluoxetine and other targeted chiral illicit drugs. This is due to the fact that metabolic processes in humans are stereoselective and lead to changes of chiral signature of excreted drugs when compared to their unused counterparts)

(iv) verification of the potency of the abused drug (e.g. *S*-(+)- enantiomers of amphetamine and methamphetamine are known to be much more potent than *R*-(-) enantiomers of the same drugs).

The developed and validated method enabled the identification, detection and quantification of most targeted human biomarkers in wastewater. The method was applied in a one week monitoring study of a large wastewater treatment plant in the UK. Wastewater profiling of 56 biomarkers was undertaken. These are: opioid analgesics, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, PDE5 inhibitors, amphetamine and methamphetamine drug precursors (Table 3-1). To the author's knowledge, this is the first time chiral separation and then wastewater profiling of mephedrone, MDMA and its metabolites HMA, HMMA, PMA (*para*-methoxyamphetamine) using chiral CBH-HPLC-MS/MS method has been reported. The latter compound is a phenylisopropylamine with hallucinogenic properties, responsible, alongside *N*-monomethyl analogue (PMMA), for several deaths due to its abuse [14-16]. Moreover, the method was applied for investigating the in-sewer stability of the targeted biomarkers in a pressurized sewer under anaerobic conditions. This stability study was performed for evaluating any alteration in concentration of the biomarker during its transport in the sewer system to the collection site. This evaluation at enantiomeric level was performed for the first time.

## 3.3 Experimental

### 3.3.1 Chemicals and materials

The following analytes were selected for the study (Table 3-1): opioid analgesics, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, PDE5 inhibitors, amphetamine and methamphetamine drug precursors. Table S1 shows all target analytes, their CAS number, molecular formula, molecular weight, $pK_a$ and supplier information.

**Table 3-1** Selected chiral drug biomarkers and their pharmacokinetic data.

| Group | Drug | Metabolite | Excretion | Source of excretion (range) |
|---|---|---|---|---|
| Stimulants | Cocaine | Cocaine | 1.0-9.0%, 7.5% | [17]; [18] |
| | | Benzoylecgonine | 32.5% (nasal) | [19] |
| | | Anhydroecgonine methyl ester (AEME) | 0.7% | [20] |
| | Cocaine and alcohol | Cocaethylene | | Drugbank [21], [20] |
| Stimulants | Amphetamine | Amphetamine | 30.0% in neutral condition of pH, up to 74.0% in acidic and 1.0% in alkaline urines | [17] |
| | | Norephedrine | 2.0% in neutral condition of pH | [17] |
| Stimulants | Methamphetamine | Methamphetamine | 43.0% at pH range between 6 and 8, up to 76.0% in acidic and 2.0% in alkaline urines | [17] |
| | | Amphetamine | 4.0-7.0% at pH range between 6 and 8 | [17] |
| Stimulants | Mephedrone | Mephedrone | Not available data | |
| Hallocinogens | MDA | MDA | Unchanged (overdose case) | [22] |
| Hallocinogens | MDMA | MDMA | 15.0% | [23] |
| | | MDA | 1.5% | [23] |
| | | DHMA | Minor | [24] |
| | | HMMA | 20.0% | [23] |
| | | HMA | 1.0% | [23] |
| Hallocinogens | MDEA | MDEA | 19.0% | [17] |
| | | MDA | 28.0% | [17] |
| Opioids | Diamorphine | Diamorphine | 0.1% | [25] |
| | | Morphine derivative (*O*-6-MAM) | 50.0-60.0% | [25] |
| Opioids | Morphine | Morphine | 10.0% | [17] |
| | | Morphine-3-glucuronide | 75.0% | [17] [26] |
| | | Hydromorphone (not targeted) | Trace | [26] |
| | | Normorphine | Not found | (Doris Clouet 2012) |
| Dissociative agent | Ketamine | Ketamine | 2.3% | [17] |
| | | Norketamine | 1.6% | [17] |
| Stimulants | Benzylpiperazine | Benzylpiperazine | 3.0-6.0% | [27] |
| Benzodiazepines | Temazepam | Temazepam | 1.5%+73.0% as conjugated | [17] |
| | | Oxazepam | 1.0%+5.8% as conjugated | [17] |
| Benzodiazepines | Diazepam | Diazepam | Trace | [17] |
| | | Oxazepam gluc | 33.0% | [17] |

| | | | | |
|---|---|---|---|---|
| | | Temazepam | 6.0% | [17] |
| | | Nordiazepam | Trace | (Steven B. Karch, 2007) |
| Benzodiazepines | Nitrazepam | Nitrazepam | trace, 1.0% (in the 7 day urine) | [17] |
| | | 7-amino-nitrazepam | 31.0% (in the 7 day urine) | [17] |
| Benzodiazepines | Oxazepam | Oxazepam | trace+61.0% as glucuronide | [17] |
| Benzodiazepines | Lorazepam | Lorazepam | trace as unchanged + 75.0% lorazepam glucuronide | [17] |
| Population biomarkers | Caffeine | Caffeine | 0.7-0.9% | |
| | | 1,7-dimethylxanthine | 14.0% | [28] |
| Population biomarkers | Nicotine | Nicotine | 13.0%, 5.0% | [5], [17] |
| | | Cotinine | 30.0%, 10.0% | [5], [17] |
| Population biomarkers | Creatinine | | | |
| Opioids | Codeine | Codeine | 10.0%, 32.0-46.0% as glucuronide | Drugbank [21], [26] |
| | | Morphine | 5.0-13.0% | [26] |
| Opioids | Oxycodone | Oxycodone | 13.0-19.0%+7.0-29.0% as conjugated | [17] |
| | | Oxymorphone | 13.0-14.0% as conjugated | [17] |
| | | Noroxycodone | Trace | [17] |
| Opioids | Hydrocodone | Hydrocodone | | |
| | | Hydromorphone (not targeted) | 5.0% | [26] |
| Opioids | Dihydrocodeine | Dihydrocodeine | 31.0%, 28.0% as conjugated | [17] |
| | | Dihydromorphine | 8.4% as conjugated | [17] |
| Opioids | Methadone | Methadone | 27.5 (5-50) | [22] |
| | | EDDP | 3.0-25.0% | [17] |
| Antidepressants | Venlafaxine | Venlafaxine | 5.0% | [17] |
| | | *O*-desmethylvenlafaxine | 29.0-48.0% | [17] |
| PDE5 Inhibitor | Vardenafil | Vardenafil | <10.0% | (Thomas L. Lemke, David A. Williams 2012) |
| Precursors | Ephedrine | Ephedrine | 70.0-80.0% | [17] |
| | | Norephedrine | 4.0% | [17] |
| | Pseudoephedrine | Pseudoephedrine | 88.0% | [17] |
| Stimulants | PMA | | | |
| Opioids | Tramadol | Tramadol | 29.0% | [17] |
| | | *O*-desmethyltramadol | 20.0% as free and conjugated | [17] |
| Z-drugs | Zolpidem | Zolpidem | Nd | [17] |
| Antidepressants | Amitriptyline | Amitriptyline | Trace | Drugbank [21] |
| PDE5 Inhibitor | Sildenafil | Sildenafil | 13.0% | [17] |

| | | | | |
|---|---|---|---|---|
| Z-drugs | Zopiclone | Zopiclone | 4.5 | [17] |
| Antidepressants | Fluoxetine | Fluoxetine | 2.5-5.0% | [29] |
| | | Norfluoxetine | 10.0% | [29] |

The following deuterated analogues of target analytes were used as internal standards (IS): cocaine-$D_3$, benzoylecgonine-$D_8$, cocaethylene-$D_3$, ecgonine methyl ester-$D_3$, amphetamine-$D_5$, methamphetamine-$D_5$, phencyclidine-$D_5$, mephedrone-$D_3$, MDA-$D_5$, MDMA-$D_5$, MDEA-$D_5$, cotinine-$D_3$, EDDP-$D_3$, heroin-$D_9$, codeine-$D_6$, oxycodone-$D_6$, hydrocodone-$D_6$, morphine-$D_6$, morphine-3β-*D*-glucuronide-$D_3$, methadone-$D_9$, temazepam-$D_5$, diazepam-$D_5$, nordiazepam-$D_5$, nitrazepam-$D_5$, oxazepam-$D_4$, lorazepam-$D_4$, zopiclone-$D_4$, ketamine-$D_4$, norketamine-$D_4$ and *1S,2R*-(+)-ephedrine-$D_3$.

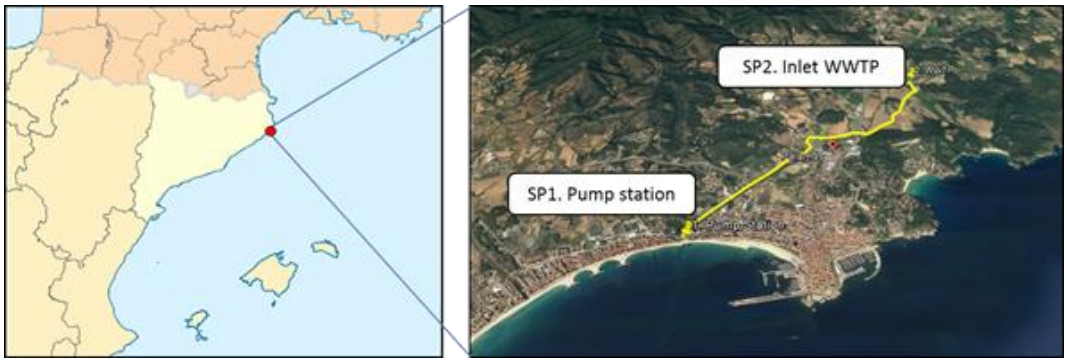
The following analytes were used as racemates: (±)-mephedrone, (±)-4-hydroxy-3-methoxymethamphetamine (HMMA), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-4-hydroxy-3-methoxyamphetamine (HMA), (±)-methamphetamine, (±)-amphetamine, (±)-3,4-methylenedioxyamphetamine (MDA), (±)-tramadol, (±)-desmethylvenlafaxine, (±)-venlafaxine, (±)-3,4-methylenedioxy-*N*-ethyl-amphetamine (MDEA), (±)-ephedrine, (±)-pseudoephedrine, (±)-*para*-methoxyamphetamine (PMA), (±)-norephedrine, (±)-norfluoxetine, (±)-zopiclone, (±)-fluoxetine, (±)-3,4-dihydroxymethamphetamine (DHMA), (±)-methadone, (±)-ketamine, (±)-norketamine, (±)-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), (±)-lorazepam, (±)-temazepam, (±)-oxazepam. Enantiomerically pure standard solutions were used for the following analytes: 6-monoacetylmorphine with five defined stereocentres; oxycodone with four defined stereocentres, also known as (-)-oxycodone; morphine-3β-*D*-glucuronide with ten defined stereocentres; hydrocodone with four defined stereocentres; dihydromorphine with five defined stereocentres; codeine, also known as (-)-codeine with five defined stereocentres; morphine, also known as *D*-(-)-morphine with four defined stereocentres; normorphine with five defined stereocentres; heroin with five defined stereocentres; dihydrocodeine, also known as (-)-dihydrocodeine with five defined stereocentres; noroxycodone with four defined stereocentres; oxymorphone, also known as (-)-oxymorphone with four defined stereocentres; cocaethylene with four defined stereocentres; cocaine, also known as (-)-cocaine with four defined stereocentres; benzoylecgonine, also known as (-)-benzoylecgonine with four defined stereocentres and anhydroecgonine methyl ester (AEME) with two defined stereocentres. All standards and internal standards were of the highest purity available (>97%). Stock and working solutions of standards were stored at -20° C. Methanol, acetonitrile and ammonium acetate were purchased from Sigma Aldrich,

UK. Ultrapure water was obtained from PURELAB UHQ-PS Unit (Elga, UK). The deactivation of the glassware was carried out in order to prevent the adsorption of polar compounds to the hydroxyl sites on the glass surface. The process consisted of the following steps: rinsing of the glassware with 5% dimethyldichlorosilane (DMDCS) once, with toluene twice and with methanol thrice.

### 3.3.2 Sample collection, storage and sample preparation

24h composite wastewater influent samples were collected in polytetrafluoroethylene (PTFE) bottles from a local wastewater treatment plant. In details, 10 mL aliquots were taken every 15 minutes by ISCO 3700 device (time-proportional sampling). They were then transported to the laboratory in cool boxes packed with ice blocks and filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK). 100 µL of a mixture of internal standard at concentration 1 mg $L^{-1}$ were added to 100 mL of a wastewater sample to give final concentration of 1 µg $L^{-1}$. Stability of analytes in wastewater has been already investigated in previous works from the group [30, 31]. For the in sewer transport stability study, triplicate samples were collected from a real pressurized sewer working under anaerobic conditions in the municipality of Palamós in the North-East coast of Catalonia (Spain) (Figure 3-1). Sampling was undertaken at the inlet (SP1) and at the outlet (SP2) of the pipe for three consecutive days for a total of 6 samples in September 2015 during dry weather conditions. Samples were collected employing two portable automatic refrigerated samplers Hach-Lange Buhler BL 2000 with 24 PE containers of 1 L located at SP1 and SP2. Samples were all prepared as discussed above. Cartridges were then sent to the laboratory in the UK, where the elution of the analytes and their analysis were performed according to the method described below. Solid phase extraction (SPE) was carried out using Oasis HLB cartridges (60 mg, Waters, UK) and the following procedure. The cartridges were conditioned with 2 mL of methanol followed by equilibration with 2 mL of ultrapure water at a rate of 3 mL $min^{-1}$. 100 mL of environmental sample (spiked with ISs at 1 µg $L^{-1}$) were passed through the HLB cartridge at a rate of 8 mL $min^{-1}$. The cartridges were then washed with 3 mL of ultrapure water at a rate of 3 mL $min^{-1}$ and the analytes were eluted with 4 mL of methanol at a rate of 8 mL $min^{-1}$ into 5 mL silanised glass tubes. The extract was transferred to the TurboVap evaporator (Caliper, UK).

**Figure 3-1** Location and layout of the studied sewer. Point 1: SP1 pump station collecting wastewater of the suburb of South-Palamós pushing it to the SP2; point 2: SP2 Palamós wastewater treatment plant.




| | |
|---|---|
| Length of the pipe | 4800 m |
| Diameter of the pipe | 556 mm |
| Hydraulic retention time | 9-15 hours |
| Average daily flow | 2812 ± 77$m^3$ sewage/day |
| Sampling | Composite samples of wastewater collected during 24-hour period using a flow-proportional sampling mode following the sampling guidelines proposed by Ort et al. (2010). |

After evaporation to dryness under nitrogen flow (5-10 psi) at 40°C the samples were reconstituted with 0.5 mL 1mM ammonium acetate/methanol 85:15 v/v and filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm). The filtered samples were transferred to polypropylene plastic vials bonded pre-slit PTFE/Silicone septa (Waters, UK) and then 20 µL were directly injected into a UHPLC-MS/MS system. Samples from monitoring campaign were prepared in duplicate and analysed twice.

### 3.3.3 Sample analysis with chiral liquid chromatography coupled with tandem mass spectrometry

Separation of all analytes was undertaken with Waters ACQUITY UPLC® system (Waters, Manchester, UK). Three chiral columns were evaluated in this study:

(1) CHIRALPAK® CBH HPLC Column 5 µm particle size, L × I.D. 10 cm × 2.0 mm (Chiral Technologies, France) with a Chiral-CBH guard column 10 × 2.0 mm, 5 µm particle size (Chiral Technologies, France);

(2) CHIROBIOTIC V column 5 μm particle size, L × I.D. 25 cm × 2.1 mm (Sigma Aldrich, UK) with a guard column 2 cm × 4.0 mm, 5 μm particle size (Sigma Aldrich, UK);

(3) CHIROBIOTIC T column 5 μm particle size, L × I.D. 25 cm × 2.1 mm (Sigma Aldrich, UK) with a guard column 2 cm × 4.0 mm 5 μm particle size (Sigma Aldrich, UK).

ACQUITY UPLC™ autosampler was kept at 4°C, while the column temperature was set at 25°C. The injection volume of the sample was 20 μL. Several mobile phase compositions were tested (see for details Tables S2, S3 and S4). Different flow rates were also trialled: 0.075 mL $min^{-1}$ and 0.1 mL $min^{-1}$. The selected chiral column was the CHIRALPAK® CBH HPLC column. The chosen mobile phase used in the method was 1mM ammonium acetate/methanol 85:15 v/v at a 0.1 mL $min^{-1}$ under isocratic conditions.

All analytes were identified and quantified using a triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an electrospray ionisation source (ESI). Analyses were performed in positive mode with an optimised capillary voltage of 3 kV, source temperature of 150°C, desolvation temperature of 265°C and desolvation gas flow of 550 l $h^{-1}$. Nitrogen, supplied by a high purity nitrogen generator (Peak Scientific, UK), was used as a nebulising and desolvation gas. Argon (99.999%) was used as a collision gas. MassLynx 4.1 (Waters, UK) was used to control the Waters ACQUITY system and the Xevo TQD. Data processing was carried out on TargetLynx software (Waters, Manchester, UK).

The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode measuring the fragmentation of the protonated pseudo-molecular ions of each compound. The choice of fragmentation ion for each compound was based on the most intense signal. MRM transitions as well as cone voltages and collision energies were obtained after direct infusion of each standard at a concentration of 100 μg $L^{-1}$ into the mass spectrometer. In the final stage of the method development, once CBH column was chosen, cone voltages and collision energies were optimised for the chosen MRM transitions through infusion of each standard at 100 μg $L^{-1}$ combined with LC using 1mM ammonium acetate/methanol 85:15 v/v as mobile phase at 0.1 mL $min^{-1}$ under isocratic conditions. Two or three MRM transitions were selected for each compound. The most abundant transition product ion was

typically used for quantification, whilst second and third transitions used for confirmation purposes for nearly all compounds. The MRM transitions of the studied compounds, cone voltages and collision energies are presented in Table 3-2.

**Table 3-2** MRM transitions selected for studied analytes.

| Compound | CV/CE[a] | MRM1 (quantification) | CV/CE[a] | MRM2 (confirmation) | CV/CE[a] | MRM3 (confirmation) | MRM1/MRM2 ratio ± SD | MRM1/MRM3 ratio ± SD | Internal standard |
|---|---|---|---|---|---|---|---|---|---|
| Cocaine | 40/20 | 304.2 > 182.1 | 40/31 | 304.2 > 82.1 | - | - | 2.8 ± 0.2 | - | Cocaine-D3 |
| Benzoylecgonine | 38/19 | 290.2 > 168.1 | 38/30 | 290.2 > 105.1 | - | - | 1.9 ± 0.2 | - | Benzoylecgonine-D8 |
| Cocaethylene | 38/20 | 318.2 > 196.2 | 38/30 | 318.2 > 82.1 | - | - | 1.9 ± 0.1 | - | Cocaethylene-D3 |
| Anhydroecgonine methyl ester (AEME) | 39/23 | 182.1 > 118.0 | 39/21 | 182.1 > 122.1 | - | - | 1.2 ± 0.1 | - | Cocaine-D3 |
| Amphetamine | 18/16 | 136.16 > 91.1 | 18/8 | 136.16 > 119.1 | - | - | 1.2 ± 0.1 | - | Amphetamine-D5 |
| Methamphetamine | 24/19 | 150.2 > 91.1 | 24/10 | 150.2 > 119.1 | - | - | 1.8 ± 0.1 | - | Methamphetamine-D5 |
| Benzylpiperazine (BZP) | 35/20 | 177.1 > 91.1 | 35/15 | 177.1 > 85.1 | - | - | 6.5 ± 0.6 | - | PCP-D5 |
| MDA | 21/11 | 180.0 > 163.1 | 21/22 | 180.0 > 105.1 | - | - | 2.6 ± 0.4 | - | MDA-D5 |
| MDMA | 24/13 | 194.1 > 163.1 | 24/24 | 194.1 > 105.1 | - | - | 2.1 ± 0.1 | - | MDMA-D5 |
| MDEA | 28/13 | 208.1 > 163.1 | 28/27 | 208.1 > 105.1 | - | - | 2.1 ± 0.2 | - | MDEA-D5 |
| HMA | 6/14 | 182.1 > 165.0 | 6/24 | 182.1 > 105.0 | 6/18 | 182.1 > 133.0 | 1.8 ± 0.7 | 2.4 ±1.4 | Amphetamine-D5 |
| HMMA | 16/12 | 196.1 > 165.0 | 16/26 | 196.1 > 105.0 | 16/22 | 196.1 > 133.0 | 3.1 ± 0.6 | 3.8 ±0.6 | Methamphetamine-D5 |
| DHMA | 6/12 | 182.1 > 151.0 | 6/18 | 182.1 > 123.0 | 6/24 | 182.1 > 105.0 | 2.8 ± 0.5 | 3.2 ±0.7 | Amphetamine-D5 |
| Mephedrone | 10/12 | 178.1 > 160.1 | 10/22 | 178.1 > 145.0 | 10/22 | 178.1 > 119.0 | 1.6 ± 0.2 | 8.5 ±2.1 | Mephedrone-D3 |
| *p*-Methoxyamphetamine (PMA) | 20/20 | 166.0 > 121.0 | 20/20 | 166.0 > 149.0 | - | - | 12.5 ± 1.5 | - | MDA-D5 |
| Heroin | 51/50 | 370.2 > 165.1 | 51/29 | 370.2 > 268.1 | - | - | 1.5 ± 0.2 | - | Heroin-D9 |
| *O*-6-monoacetylmorphine (*O*-6-MAM) | 52/39 | 328.1 > 165.1 | 52/26 | 328.1 > 211.1 | - | - | 1.4 ± 0.3 | - | PCP-D5 |
| Codeine | 49/25 | 300.2 > 215.1 | 49/57 | 300.2 > 152.1 | - | - | 1.8 ± 0.1 | - | Codeine-D6 |
| Oxycodone | 36/29 | 316.2 > 241.1 | 36/26 | 316.2 > 256.1 | - | - | 1.4 ± 0.3 | - | Oxycodone-D6 |
| Noroxycodone | 22/36 | 302.1 > 227.0 | 22/28 | 302.1 > 187.0 | - | - | 5.5 ± 0.8 | | Oxycodone-D6 |
| Hydrocodone | 24/34 | 300.1 > 199.0 | 24/46 | 300.1 > 171.0 | - | - | 3.7 ± 0.2 | - | Hydrocodone-D6 |
| Oxymorphone | 40/19 | 302.1 > 284.1 | 40/28 | 302.1 > 227.1 | - | - | 2.3 ± 0.2 | - | Oxycodone-D6 |
| Morphine | 53/38 | 286.1 > 165.1 | 53/56 | 286.1 > 152.1 | - | - | 1.2 ± 0.2 | - | Morphine-D6 |
| Normorphine | 45/43 | 272.1 > 165.0 | 45/49 | 272.1 > 152.1 | - | - | 1.3 ± 0.5 | - | Morphine-D6 |
| Dihydromorphine | 28/42 | 288.2 > 185.0 | 28/32 | 288.2 > 213.0 | 28/42 | 288.2 > 231.0 | 2.9 ± 0.5 | 129.6 ±68.4 | Morphine-D6 |
| Dihydrocodeine | 53/33 | 302.1 > 199.1 | 53/60 | 302.1 > 128.1 | - | - | 1.9 ± 0.2 | - | Codeine-D6 |
| Morphine-3β-*D*-glucuronide | 56/44 | 462.3 > 286.1 | 56/80 | 462.3 > 165.0 | 56/56 | 462.3 > 201.1 | 4.7 ± 1.8 | 9.88 ±3.0 | Morphine-3β-D-glucuronide-D3 |
| Methadone | 31/15 | 310.2 > 265.1 | 31/28 | 310.2 > 105.1 | - | - | 1.6 ± 0.5 | - | Methadone-D9 |
| EDDP | 50/29 | 278.2 > 234.1 | 50/24 | 278.2 > 249.1 | - | - | 2.3 ± 0.1 | - | EDDP-D3 |
| Tramadol | 24/17 | 264.2 > 58.1 | 24/11 | 264.2 > 246.3 | - | - | 102.1 ± 3.6 | - | Methamphetamine-D5 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *O*-desmethyl-tramadol | 2/18 | 250.1 > 58.0 | 2/20 | 250.1 > 232.0 | 2/34 | 250.1 > 107.0 | 899.7 ± 14.7 | 1228.0 ±373.0 | Codeine-D6 |
| Temazepam | 37/21 | 301.1 > 255.1 | 37/14 | 301.1 > 283.1 | - | - | 2.2 ± 0.1 | - | Temazepam-D5 |
| Diazepam | 54/27 | 285.0 > 154.1 | 54/31 | 285.0 > 193.1 | - | - | 1.2 ± 0.1 | - | Diazepam-D5 |
| Nordiazepam | 51/29 | 271.1 > 140.1 | 51/29 | 271.1 > 165.0 | - | - | 2.0 ± 0.1 | - | Nordiazepam-D5 |
| Nitrazepam | 44/24 | 282.1 > 236.1 | 44/37 | 282.1 > 180.1 | - | - | 2.5 ± 0.3 | - | Nitrazepam-D5 |
| 7-aminonitrazepam | 48/25 | 252.1 > 121.1 | 48/40 | 252.1 > 94.1 | - | - | 5.7 ± 0.9 | - | Nitrazepam-D5 |
| Oxazepam | 38/21 | 287.1 > 241.1 | 38/15 | 287.1 > 269.0 | - | - | 1.3 ± 0. | - | Oxazepam-D4 |
| Lorazepam | 30/20 | 321.0 > 275.1 | 30/33 | 321.0 > 229.1 | - | - | 3.3 ± 1.4 | - | Lorazepam-D4 |
| Zopiclone | 22/18 | 389.1 > 245.0 | 22/42 | 389.1 > 217.0 | - | - | | | Zopiclone-D4 |
| Zolpidem | 8/36 | 308.2 > 235.2 | 8/36 | 308.2 > 263.0 | - | - | 1.7 ± 0.5 | | Cocaine-D3 |
| Amitriptyline | 37/26 | 278.2 > 91.1 | 37/18 | 278.2 > 233.2 | - | - | 1.8 ± 0.2 | - | EDDP-D3 |
| Fluoxetine | 25/8 | 310.3 > 148.1 | - | - | - | - | - | - | MDMA-D5 |
| Norfluoxetine | 17/7 | 296.2 > 134.1 | - | - | - | - | - | - | MDMA-D5 |
| Venlafaxine | 27/12 | 278.2 > 58.1 | 27/12 | 278.2 > 260.1 | 27/32 | 278.2 > 121.0 | 2.7 ± 0.2 | 4.5 ±0.7 | Methamphetamine-D5 |
| Desmethylvenlafaxine | 25/24 | 264.0 > 58.1 | 25/24 | 264.0 > 107.1 | 25/20 | 264.0 > 246.3 | 12.7 ± 1.8 | 66.4 ±5.9 | Methamphetamine-D5 |
| Ketamine | 31/27 | 238.1 > 125.0 | 31/15 | 238.1 > 220.1 | - | - | 3.3 ± 0.5 | - | Ketamine-D4 |
| Norketamine | 23/27 | 224.0 > 125.0 | 23/12 | 224.0 > 207.1 | - | - | 1.1 ± 0.1 | - | Norketamine-D4 |
| Sildenafil | 60/28 | 475.3 > 100.2 | 68/50 | 475.3 > 283.2 | 68/36 | 475.3 > 311.2 | 28.6 ± 8.6 | 17.3 ±4.0 | PCP-D5 |
| Vardenafil | 74/68 | 489.3 > 151.0 | 74/48 | 489.3 > 321.1 | - | - | 7.2 ± 3.0 | - | Methadone-D9 |
| Ephedrine | 23/12 | 166.1 > 148.1 | 23/21 | 166.1 > 133.0 | - | - | 7.4 ± 0.8 | - | *1S, 2R*-(+)-ephedrine-D3 |
| Pseudoephedrine | 23/12 | 166.1 > 148.1 | 23/21 | 166.1 > 133.0 | - | - | 6.9 ± 0.6 | - | *1S, 2R*-(+)-ephedrine-D3 |
| Norephedrine | 23/10 | 152.1 > 134.1 | 23/16 | 152.1 > 117.1 | - | - | 3.1 ± 0.4 | - | *1S, 2R*-(+)-ephedrine-D3 |
| Caffeine | 38/15 | 195.1 > 138.0 | 38/23 | 195.1 > 110.0 | - | - | 2.5 ± 0.3 | - | Cotinine-D3 |
| 1,7-dimethylxanthine | 54/21 | 181.0 > 124.1 | - | - | - | - | - | - | Cotinine-D3 |
| Nicotine | 37/20 | 163.1 > 130.0 | 37/24 | 163.1 > 117.0 | - | - | 1.4 ± 0.1 | - | Cotinine-D3 |
| Cotinine | 34/21 | 177.1 > 80.0 | 34/22 | 177.1 > 98.1 | - | - | 2.8 ± 0.2 | - | Cotinine-D3 |
| Creatinine | 31/11 | 114.0 > 86.1 | 31/16 | 114.0 > 72.1 | - | - | 21.9 ± 4.2 | - | Cotinine-D3 |

[a]CV, cone voltage (V); CE, collision energy (eV)

Selection of ISs (see Table 3-3) for those compounds for which deuterated or $C^{13}$ analogues were not available commercially or in our laboratory was based on structural similarity and elution time to account for possible signal suppression/enhancement of studied analytes in ESI.

**Table 3-3** MRM transitions selected for IS standards used in the method.

| ISs | CV/CE[a] | MRM1 (quantification) | Supplier |
|---|---|---|---|
| Cocaine-$D_3$ | 40/20 | 307.2 > 185.1 | Cerilliant |
| Benzoylecgonine-$D_8$ | 38/19 | 298.2 > 171.1 | Cerilliant |
| Cocaethylene-$D_3$ | 42/20 | 321.2 > 199.1 | Cerilliant |
| Ecgonine methyl ester-$D_3$ | 44/22 | 203.2 > 185.2 | Cerilliant |
| Amphetamine-$D_5$ | 22/16 | 141.0 > 92.9 | Cerilliant |
| Methamphetamine-$D_5$ | 28/12 | 155.1 > 121.0 | Cerilliant |
| PCP-$D_5$ | 18/14 | 249.2 > 164.1 | Cerilliant |
| Mephedrone-$D_3$ | 30/22 | 181.1 > 163.1 | Cerilliant |
| MDA-$D_5$ | 21/11 | 185.1 > 168.1 | Cerilliant |
| MDMA-$D_5$ | 26/13 | 199.1 > 165.1 | Cerilliant |
| MDEA-$D_5$ | 28/13 | 213.1 > 163.0 | Cerilliant |
| Cotinine-$D_3$ | 44/24 | 180.1 > 80.0 | Cerilliant |
| EDDP-$D_3$ | 50/29 | 281.2 > 234.1 | LGC Standards |
| Heroin-$D_9$ | 51/50 | 379.2 > 165.8 | Cerilliant |
| Codeine-$D_6$ | 52/28 | 306.2 > 218.1 | Cerilliant |
| Oxycodone-$D_6$ | 36/29 | 322.2 > 247.1 | Cerilliant |
| Hydrocodone-$D_6$ | 64/32 | 306.2 > 202.0 | Cerilliant |
| Morphine-$D_6$ | 53/38 | 292.2 > 153.1 | Cerilliant |
| Morphine-3β-D-glucuronide-$D_3$ | 52/36 | 465.2 > 289.1 | Cerilliant |
| Methadone-$D_9$ | 31/15 | 319.3 > 268.2 | Cerilliant |
| Temazepam-$D_5$ | 37/21 | 306.7 > 260.1 | Cerilliant |
| Diazepam-$D_5$ | 54/27 | 290.1 > 154.1 | Cerilliant |
| Nordiazepam-$D_5$ | 48/36 | 276.1 > 140.1 | Cerilliant |
| Nitrazepam-$D_5$ | 52/42 | 287.1 > 185.0 | Cerilliant |
| Oxazepam-$D_4$ | 38/21 | 292.0 > 246.0 | Cerilliant |
| Lorazepam-$D_4$ | 25/29 | 325.0 > 279.2 | Cerilliant |
| Zopiclone-$D_4$ | 24/16 | 393.1 > 245.0 | Cerilliant |
| Ketamine-$D_4$ | 31/27 | 242.1 > 129.1 | Cerilliant |
| Norketamine-$D_4$ | 32/28 | 228.1 > 128.9 | Cerilliant |
| *1S,2R*-(+)-Ephedrine-$D_3$ | 23/18 | 169.2 > 151.0 | LGC Standards |

[a]CV, cone voltage (V); CE, collision energy (eV)

### 3.3.4 Method Validation

The developed method was fully validated for wastewater samples. The following parameters were studied: instrumental and method limits of detection and quantification, linearity, precision and accuracy, ion suppression, resolution of enantiomers and enantiomeric fraction. Due to the potential presence of target analytes in wastewater deuterated analogues of the targeted analytes were used as internal standards and to evaluate method performance.

The instrumental limit of detection (IDL) was determined at a concentration value giving a signal-to-noise ratio (S/N) ≥ 3 for all the MRM transitions selected for each substance. The method detection limit (MDL) was calculated using the following formula:

$$\mathrm{MDL} = \frac{(\mathrm{IDL} \times 100)}{\mathrm{Rec} \times \mathrm{CF}} \quad (4)$$

where Rec is the relative SPE recovery of the analyte in the matrix and CF is the SPE concentration factor.

The instrumental limit of quantification (IQL) was determined at the minimum concentration value giving S/N ≥ 10 for all the MRM transitions. The method quantification limit (MQL) was calculated using the following formula:

$$\mathrm{MQL} = \frac{(\mathrm{IQL} \times 100)}{\mathrm{Rec} \times \mathrm{CF}} \quad (5)$$

The linearity of the method was verified for each compound in the following range: IDL - 1000 µg $L^{-1}$. The individual calibrators were at a concentration of 1000, 800, 700, 600, 500, 400, 300, 200, 100, 50, 10, 5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005 and 0 µg $L^{-1}$.

For some compounds, especially human indicators, such as caffeine and creatinine, dilution integrity was considered as these substances are present at high concentrations in wastewater in respect to the illicit drugs concentration range. Dilution integrity was assessed through the analysis of two diluted samples 1:10 and 1:100 at the highest concentration in wastewater spiked with a mixture of ISs. If the compound could be quantified with a relative error within the 15% in relation to the nominal concentration, the dilution integrity was maintained.

Precision, expressed as relative standard deviation (RSD) of replicate analysis ($n$=4) at three different concentrations on the same day (intra-RSD%), was evaluated as:

(i) instrumental precision using standard solutions spiked in mobile phase at 10, 100 and 1000 µg $L^{-1}$ for (non-chiral/not enantiomerically separated) analytes, or at 5, 50 and 500 µg $L^{-1}$ for individual enantiomers (separated from racemic mixture);

(ii) method precision using standard solutions spiked in 100 mL of influent wastewater at 50, 500 and 5000 ng $L^{-1}$ for (non-chiral/not enantiomerically separated) analytes, or at 25, 250 and 2500 ng $L^{-1}$ for individual enantiomers

(separated from racemic mixture). The extraction by SPE of these samples followed the same protocol described in 3.3.2.

Reproducibility (inter-day precision) of the method was determined by replicate measurements ($n$=3) of the same concentrations of analytes as in the case of intra-day precision on three different days in order to assess the inter-day instrumental precision and the inter-day method precision. Precision data were acceptable when the RSD% was less than 15% for all the concentrations investigated during the different days.

Accuracy of the method was expressed as percentage of closeness agreement between the mean of a set of analytical results and the theoretical value.

Carryover was studied by injecting a spiked sample at a concentration of 1000 µg $L^{-1}$ followed by three blanks and it was considered insignificant if the concentration of the analyte was below the LOQ.

Ion suppression was calculated for each analyte as a percentage decrease in signal intensity in a sample matrix versus in mobile phase (free from analytes). Signal suppression was calculated using the following equation:

$$\text{Signal suppression}\,[\%] = \left(1 - \frac{\text{Is} - \text{Io}}{\text{I}_{\text{MP}}}\right) * 100 \qquad (6)$$

where $I_S$ was the analyte peak area in wastewater extract (0.5 mL) spiked after SPE extraction with 100 ng, $I_0$ was the analyte peak area in unspiked wastewater extract, $I_{MP}$ was the analyte peak area in mobile phase (0.5 mL) spiked with 100 ng of each analyte.

Resolution of enantiomers of chiral drugs ($R_s$) was calculated using the following equation:

$$R_s = \frac{2\left(t_{rE2} - t_{rE1}\right)}{\left(w_{bE2} + w_{bE1}\right)} \qquad (7)$$

where $t_{rE1}$ and $t_{rE2}$ are retention times of the first- and the second-eluted enantiomer respectively and $w_{bE1}$, $w_{bE2}$ are widths of their responses at a baseline. $R_s \geq 1.2$ indicates full baseline resolution. $R_s$ =1 indicates 2% overlap which is deemed acceptable for quantification purposes.

Enantiomeric fraction (EF) was calculated using the following equation:

$$EF = \frac{(+)}{[(+) + (-)]} \qquad (8)$$

where (+) is the concentration of (+)-enantiomer or first eluted enantiomer, and (-) is the concentration of (-)-enantiomer or second eluted enantiomer. EF equals 1 or 0 in the case of enantiomerically pure compound and 0.5 in the case of a racemate. The assessment of the absolute configuration of the first eluted or second eluted enantiomer was achieved through the injection of an enantiomerically pure standard (when available).

Validation protocols were in agreement with European Guidelines concerning the performance of analytical methods and the interpretation of results [32]).

### 3.3.5 Quantification and quality controls

The identification criteria for each analyte were as follows [32]:

- %RSD of relative retention time (RRT) should not exceed ±2.5% when compared to RRT of standard solution.
- All selected MRM transitions need to be present.
- The maximum permitted tolerance for relative ion intensities of MRM transitions should not change more than ±20% for ions with relative intensities of >50%, ±25% for ions with relative intensities between 20% and 50%, 30% for ions with relative intensities between 10% and 20% and ±50% for ions with relative intensities less than 10%.

Quality controls at 10, 100 and 1000 µg $L^{-1}$ were also prepared and injected on regular basis to maintain instrument's performance.

## 3.4 Results and Discussions

### 3.4.1 Choice of Biomarkers

Fifty six compounds were selected and targeted as potential human biomarkers of drug consumption. These are: opioid analgesics, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, PDE5 inhibitors, amphetamine and methamphetamine drug precursors (Table 3-1). Multiple human urine indicators, such as creatinine, caffeine, nicotine, 1,7-dimethylxanthine, cotinine, were also targeted as indicators of population size served by a wastewater treatment plant in question.

The selection process of target drug biomarkers included the investigation of: (i) classical drugs of abuse, with good literature based evidence of their detection and

quantification in wastewater; (ii) new emerging drugs of abuse for further study, even if prevalence data and stability data in wastewater are not well documented, and (iii) substances with abuse potential.

Cocaine, benzoylecgonine, anhydroecgonine methylester and cocaethylene were selected as biomarkers of cocaine abuse. Indeed, anhydroecgonine methylester, ethylecgonindine and ecgonidine were identified as suitable indicators of crack cocaine [33]. Moreover, cocaethylene was chosen as biomarker of co-administration of cocaine and ethanol [34]. Ecgonidine and norcocaine were not included in this study as they were not detected in a previous UK study by Baker et al. (2014). Furthermore, cuscohygrine, a marker of coca chewing [35], was also included in the method in order to distinguish between chewing of cocoa leaves (the "coqueo", a practise well-known in South America) and illegal abuse of cocaine. To the author's knowledge, no investigation of cuscohygrine and hygrine (cocoa chewing markers) has been undertaken to date. It is however worth mentioning that the practise of chewing cocaine is a non-European habit. Cuscohygrine was included in the method development but not in the method validation due to low sensitivity and poor chromatography (results are included in the supplementary data).

### 3.4.2 Method development for the detection of illicit/licit abused drugs in wastewater

#### 3.4.2.1 Chiral-CBH column

The CHIRAL-CBH column contains a protein cellobiohydrolase (CBH) as the chiral selector which is immobilised onto spherical 5 µm silica particles. The protein has a molecular weight of 60,000–70,000 and an isoelectric point of 3.9. The chiral recognition site is 4Å×7Å×40 Å-long tunnel in the core of the protein. The tunnel contains seven acidic amino acid residues, four tryptofan residues and also tyrosine, serine, threonine, arginine and histidine. The mechanism of retention of analytes in CHIRAL-CBH column can therefore involve a combination of ion exchange, hydrogen bonding and hydrophobic interactions. The enantioselectivity of the retention is regulated by the pH of mobile phase, the nature and concentration of the organic modifier and the aqueous buffer [7]. Therefore, in order to achieve the best chiral recognition of target chiral analytes within one analytical run, the following parameters were investigated in this study: type and concentration of

organic modifier (acetonitrile, methanol an isopropanol) in aqueous mobile phase and concentration of ammonium acetate.

In order to undertake quantitative measurements at enantiomeric level we aimed at obtaining enantiomeric resolution with maximum 2% overlap for each pair of enantiomers ($R_s \geq 1$). Our study revealed that $R_s \geq 1$ was achieved only in the case of 3 compounds (HMA, fluoxetine and zopiclone) in 1mM ammonium acetate/acetonitrile 9:1, 7 compounds in 1mM ammonium acetate/isopropanol 9:1 and >10 compounds in 1mM ammonium acetate/methanol 8.5:1.5 (Figures S1-3). A comparison of mobile phases with the same water content revealed that the separation selectivity differed for protic and aprotic solvents. Acetonitrile (an aprotic solvent) did not provide an adequate separation selectivity as opposed to protic solvents such as methanol and isopropanol (fluoxetine was an exception). Moreover, better separation selectivity was observed for more polar methanol than isopropanol. Furthermore, the water content in mobile phases containing isopropanol or methanol had an impact on enantioselectivity in the case of most of the studied analytes. In fact, lower water content provided higher resolution of enantiomers for tested mobile phases.

Additionally, different organic content in aqueous mobile phases affected retention times of many compounds as shown in Figure S4. A trend between retention time and the methanol content with three different concentrations of ammonium acetate was observed. Indeed, higher concentrations of ammonium acetate led to lower resolution values and lower retention times. Furthermore, retention times decreased with a higher content of methanol. In contrast, retention times increased with an increase of concentration of isopropanol (Figure S5).

The salt concentration plays a key role in controlling the pH of mobile phase, ionisation of analytes and resulting interactions between analytes and the stationary phase. In this study, ammonium acetate was used. The amphetamine-like compounds (except for PMA) showed higher resolution with lower concentration of ammonium acetate in the mobile phase. Overall, lower concentrations of ammonium acetate were preferred. However, this trend was not observed in the case of cyclopyrrolone zopiclone and the substituted cyclohexanone norketamine (Figure S3).

#### 3.4.2.2 Chirobiotic V and T

Chirobiotic V and T, two chiral columns having macrocyclic antibiotics as chiral selectors, were also tested. In reversed-phase conditions, not only cationic and anionic interactions are possible by changing pH of the mobile phase but also inclusion of the pocket and hydrogen bonding are favoured. In polar organic mode, other interactions are involved, such as dipole stacking and π-π complexation.

A comparison between CBH and Chirobiotic V columns was performed by Bagnall et al. 2012 [36]. Due to the utilisation of a combination of ion exchange, hydrogen bonding and hydrophobic interactions, CBH column was more selective in providing better enantiomeric resolution (i.e. $R_{s\ MDMA\ CBH}$ 1.9 > $R_{s\ MDMA\ CBV}$ 1.0) than Chirobiotic V. In general, CBH column provided good results in terms of separation and resolution of amphetamine-like compounds when compared to Chirobiotic V.

Experiments carried out with Chirobiotic T column showed high enantioselectivity ($R_s \geq 2.2$) for benzodiazepines only, a class of compounds not enantiomerically resolved using CBH column (Figure S5). It is worth emphasising that polar organic mobile phases, containing only methanol and 99% methanol/0.005% FA/1mM ammonium acetate, provided the best chiral recognition for most of the chiral benzodiazepines. Furthermore, mobile phases containing comparable quantities of an acid as a mobile phase additive provided better chiral recognition at lower concentrations of ammonium acetate (Figure S6).

After taking above results into consideration, the best chiral recognition for the widest group of analytes, combined with acceptable retention times, was achieved with the CBH column and a mobile phase composed of 1mM ammonium acetate/methanol 85:15 (pH 6.4).

### 3.4.3 Method validation for the detection of illicit/licit abused drugs

#### 3.4.3.1 Solid phase extraction

Oasis HLB cartridges are the sorbents of choice when utilising chiral separations with the CBH column. Relative recoveries data are reported in Table 3-4. Recoveries were high (on average > 90%) for all analysed compounds.

**Table 3-4** SPE recovery for the studied analytes.

| | SPE relative recovery % (n=3) | | |
|---|---|---|---|
| **Analyte** | 25 ng/L* | 250 ng/L* | 2500 ng/L* |
| Cocaine | 100.0 ± 1.9 | 91.0 ± 0.7 | 85.0 ± 1.7 |
| Benzoylecgonine | 76.0 ± 1.4 | 79.0 ± 1.8 | 98.0 ± 3.9 |
| Cocaethylene | 102.0 ± 2.6 | 92.0 ± 0.2 | 94.0 ± 1.4 |
| *R*-(-)-Amphetamine | 101.0 ± 6.6 | 76.0 ± 1.6 | 82.0 ± 4.7 |
| *S*-(+)-Amphetamine | 81.0 ± 10.6 | 99.0 ± 2.0 | 82.0 ± 4.2 |
| *R*-(-)-Methamphetamine | 91.0 ± 4.4 | 113.0 ± 0.7 | 82.0 ± 5.0 |
| *S*-(+)-Methamphetamine | 84.0 ± 1.9 | 86.0 ± 1.2 | 84.0 ± 7.1 |
| E1-Mephedrone | 109.0 ± 3.2 | 99.0 ± 4.8 | 80.0 ± 7.0 |
| E2-Mephedrone | 99.0 ± 8.5 | 99.0 ± 4.3 | 87.0 ± 11.5 |
| *R*-(-)-MDA | 93.0 ± 6.2 | 94.0 ± 4.2 | 81.0 ± 1.0 |
| *S*-(+)-MDA | 110.0 ± 8.5 | 99.0 ± 1.5 | 91.0 ± 1.5 |
| *R*-(-)-MDMA | 91.0 ± 3.7 | 81.0 ± 7.8 | 89.0 ± 4.3 |
| *S*-(+)-MDMA | 93.0 ± 1.7 | 100.0 ± 0.7 | 84.0 ± 1.9 |
| E1-MDEA | 102.0 ± 2.0 | 95.0 ± 8.6 | 91.0 ± 5.9 |
| E2-MDEA | 99.0 ± 1.8 | 92.0 ± 1.9 | 93.0 ± 13.4 |
| Heroin | 86.0 ± 9.4 | 80.0 ± 5.6 | 75.0 ± 2.4 |
| *O*-6-monoacetylmorphine | 108.0 ± 2.3 | 120.0 ± 1.4 | 114.0 ± 1.0 |
| Morphine | 98.0 ± 15.2 | 92.0 ± 1.3 | 112.0 ± 3.2 |
| Morphine-3β-*D*-glucuronide | 99.0 ± 0.5 | 121.0 ± 2.5 | 109.0 ± 5.3 |
| Ketamine | 127.0 ± 2.5 | 100.0 ± 5.7 | 85.0 ± 6.2 |
| Benzylpiperazine | 112.0 ± 5.3 | 96.0 ± 13.1 | 100.0 ± 4.9 |
| Temazepam | 117.0 ± 4.1 | 117.0 ± 3.0 | 99.0 ± 4.7 |
| Diazepam | 93.0 ± 3.9 | 115.0 ± 0.3 | 95.0 ± 4.9 |
| Nordiazepam | 108.0 ± 9.0 | 108.0 ± 11.2 | 96.0 ± 4.7 |
| Nitrazepam | 89.0 ± 3.1 | 91.0 ± 2.7 | 89.0 ± 1.5 |
| Oxazepam | 92.0 ± 2.7 | 117.0 ± 1.4 | 92.0 ± 1.6 |
| 7-amino-nitrazepam | 83.0 ± 6.4 | 85.0 ± 0.4 | 80.0 ± 4.1 |
| Lorazepam | 98.0 ± 14.4 | 108.0 ± 3.4 | 86.0 ± 3.0 |
| Anhydroecgonine methyl ester | 80.0 ± 5.3 | 102.0 ± 0.1 | 86.0 ± 0.4 |
| E1-HMA | 97.0 ± 8.7 | 114.0 ± 0.3 | 106.0 ± 16.4 |
| E2-HMA | 106.0 ± 4.6 | 107.0 ± 2.9 | 120.0 ± 11.5 |
| E1-HMMA | 84.0 ± 8.8 | 85.0 ± 9.4 | 100.0 ± 3.3 |
| E2-HMMA | 108.0 ± 7.5 | 105.0 ± 2.4 | 118.0 ± 1.7 |
| DHMA | 108 ± 11.9 | 112 ± 2.4 | 111 ± 2.8 |
| Caffeine | 80.0 ± 2.5 | 84.0 ± 2.7 | 80.0 ± 1.5 |
| 1,7-dimethylxanthine | 104.0 ± 0.5 | 100.0 ± 1.3 | 106.0 ± 2.4 |
| Nicotine | 97.0 ± 2.5 | 81.0 ± 6.2 | 120.0 ± 9.5 |
| Cotinine | 105.0 ± 2.8 | 93.0 ± 6.1 | 89.0 ± 3.5 |
| Creatinine | 80.0 ± 4.6 | 94.0 ± 9.5 | 109.0 ± 13.3 |
| Codeine | 95.0 ± 6.7 | 108.0 ± 3.1 | 107.0 ± 2.1 |
| Oxycodone | 84.0 ± 2.1 | 91.0 ± 3.8 | 99.0 ± 3.3 |
| Noroxycodone | 93.0 ± 11.3 | 80.0 ± 2.8 | 90.0 ± 3.2 |
| Hydrocodone | 84.0 ± 3.1 | 104.0 ± 8.9 | 101.0 ± 10.6 |
| Oxymorphone | 94.0 ± 5.7 | 87.0 ± 7.7 | 89.0 ± 1.2 |
| Dihydrocodeine | 98.0 ± 7.3 | 104.0 ± 2.9 | 89.0 ± 3.8 |
| Methadone | 95.0 ± 0.9 | 116.0 ± 0.5 | 89.0 ± 1.4 |
| EDDP | 90.0 ± 6.5 | 97.0 ± 3.0 | 90.0 ± 1.1 |
| E1-Venlafaxine | 83.0 ± 0.6 | 105.0 ± 6.3 | 91.0 ± 0.4 |
| E2-Venlafaxine | 91.0 ± 5.8 | 104.0 ± 5.4 | 90.0 ± 0.7 |
| Vardenafil | 120.0 ± 0.5 | 115.0 ± 11.0 | 100.0 ± 8.8 |
| E1-Norephedrine | 112.0 ± 2.8 | 117.0 ± 1.1 | 108.0 ± 1.5 |
| E2-Norephedrine | 115.0 ± 5.9 | 95.0 ± 2.1 | 83.0 ± 1.4 |
| E1-PMA | 110.0 ± 8.5 | 94.0 ± 2.4 | 80.0 ± 0.7 |
| E2-PMA | 113.0 ± 3.5 | 118.0 ± 5.9 | 91.0 ± 0.4 |
| Normorphine | 80.0 ± 8.4 | 80.0 ± 11.9 | 111.0 ± 4.0 |
| Dihydromorphine | 106.0 ± 0.5 | 80.0 ± 2.0 | 80.0 ± 4.5 |
| D1-Tramadol | 109.0 ± 6.0 | 111.0 ± 7.2 | 96.0 ± 10.0 |
| D2-Tramadol | 90.0 ± 7.8 | 81.0 ± 2.7 | 80.0 ± 1.1 |
| *O*-Demethyltramadol | 80.0 ± 6.4 | 118.0 ± 4.4 | 80.0 ± 3.3 |
| Zolpidem | 101.0 ± 0.8 | 96.0 ± 14.0 | 115.0 ± 1.7 |
| Amitriptyline | 81.0 ± 0.2 | 82.0 ± 2.9 | 92.0 ± 2.7 |

| | | | |
|---|---|---|---|
| Norketamine | 89.0 ± 8.2 | 116.0 ± 2.6 | 102.0 ± 2.2 |
| Sildenafil | 115.0 ± 0.7 | 105.0 ± 8.5 | 96.0 ± 9.0 |
| (+)-Ephedrine | 81.0 ± 9.0 | 82.0 ± 2.6 | 91.0 ± 2.1 |
| (-)-Ephedrine and (-)-Ψephedrine | 112.0 ± 0.6 | 87.0 ± 2.5 | 113.0 ± 9.6 |
| (+)-Ψephedrine | 104.0 ± 10.6 | 83.0 ± 0.3 | 81.0 ± 1.0 |
| Desmethylvenlafaxine-E1 | 91.0 ± 9.8 | 113.0 ± 14.2 | 98.0 ± 6.5 |
| Desmethylvenlafaxine-E2 | 82.0 ± 1.1 | 92.0 ± 4.1 | 99.0 ± 10.7 |
| E1-Zopiclone | 80.0 ± 2.0 | 82.0 ± 0.7 | 81.0 ± 3.7 |
| E2-Zopiclone | 80.0 ± 1.2 | 80.0 ± 6.7 | 83.0 ± 4.6 |
| *S*-(+)-Fluoxetine | 100.0 ± 5.5 | 81.0 ± 3.8 | 100.0 ± 0.7 |
| *R*-(-)-Fluoxetine | 97.0 ± 16.6 | 91.0 ± 5.5 | 101.0 ± 7.1 |
| E1-Norfluoxetine | 87.0 ± 1.7 | 80.0 ± 4.6 | 87.0 ± 5.3 |
| E2-Norfluoxetine | 80.0 ± 0.4 | 81.0 ± 1.7 | 84.0 ± 2.6 |

*- the following concentrations were used: 50, 500 and 5000 ng $L^{-1}$ in the case of compounds that were not enantioseparated.

### 3.4.3.2 Instrumental and method validation parameters

Figure 3-2 shows mass chromatograms of MRM 1 transitions used for quantification purposes, for each investigated analyte of a spiked influent wastewater sample at a concentration of 500 ng $L^{-1}$. The developed method allowed for identification and quantification of all studied analytes with satisfactory sensitivity and specificity.

Concentrations of compounds were calculated using the standard calibration curves which were developed using a detector response defined as the ratio of the peak ion (the specific product ion of the highest intensity, MRM1) to the base peak ion of the internal standard. The mean correlation coefficients ($R^2$) of the calibration curves were on average > 0.997 for the investigated compounds (Table 3-5).

The linearity ranges varied for different analytes. Most analytes showed linearity from 0.25 µg $L^{-1}$ up to 500 or 1000 µg $L^{-1}$ (for single enantiomer or racemate respectively). Opioids, DHMA, lorazepam, creatinine and 1,7-dimethylxanthine showed very good linearity in the range: 1 µg $L^{-1}$ - 500 or 1000 µg $L^{-1}$ (for single enantiomer or racemate respectively). Amphetamine-like compounds gave linearity from 0.125 µg $L^{-1}$ to 500 or 1000 µg $L^{-1}$ (for single enantiomer or racemate respectively) showing a high level of performance of the CBH column for these compounds. Cocaine and its metabolites responded with a linearity range of 0.01 µg $L^{-1}$ - 500 or 1000 µg $L^{-1}$ (for single enantiomer or racemate respectively). In the case of compounds present in wastewater at high concentrations exceeding accepted linearity ranges, dilution (1:10 or 1:100) of samples was utilised. It was maintained with a relative error <15%.

Good enantiomeric resolution ($R_s \geq 1.0$, allowing for quantification of individual enantiomers) was obtained for most analytes (Table 3-6).

**Figure 3-2** Chromatograms of the quantification MRM transition for each investigated analyte of a spiked influent wastewater sample at a concentration of 500 ng $L^{-1}$with CBH column.

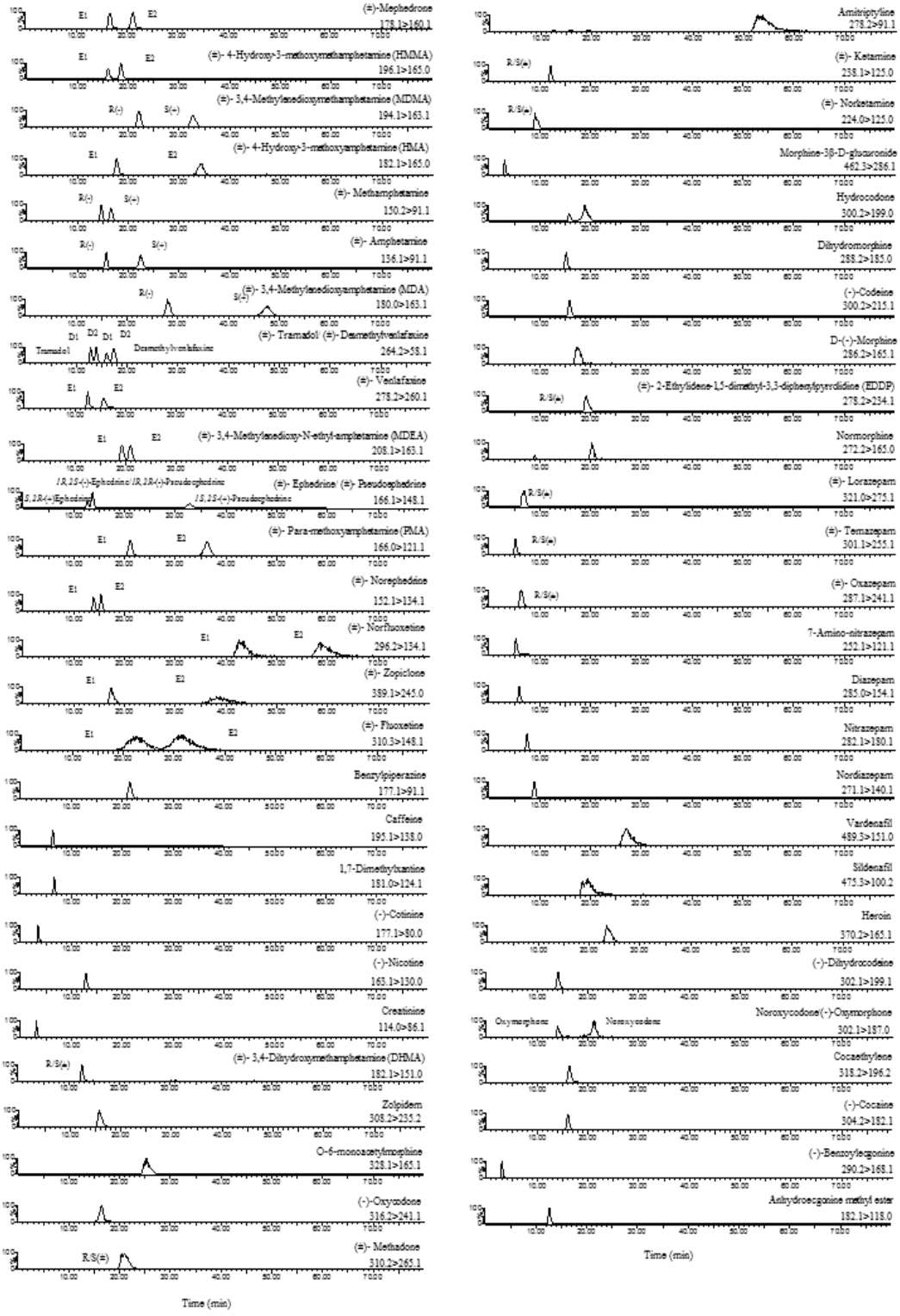
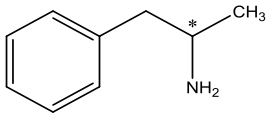
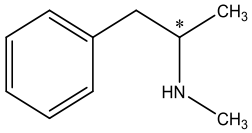
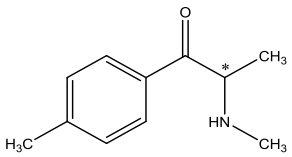
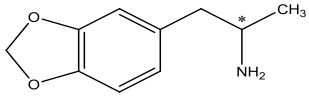
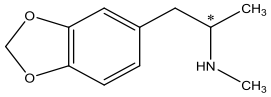
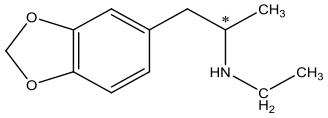
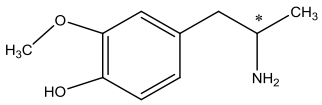
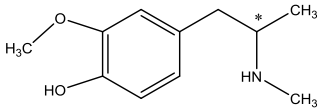
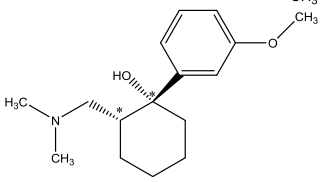
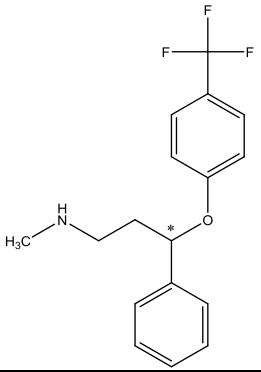


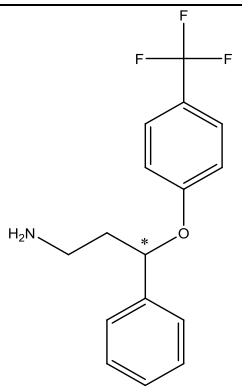
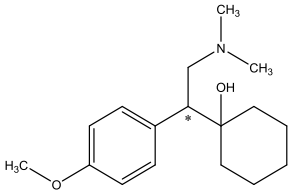
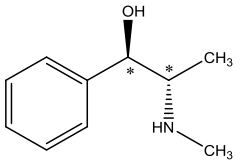
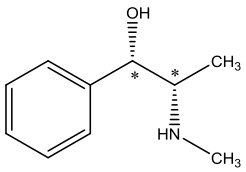
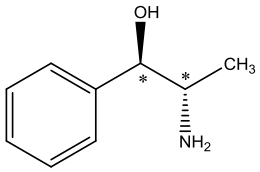
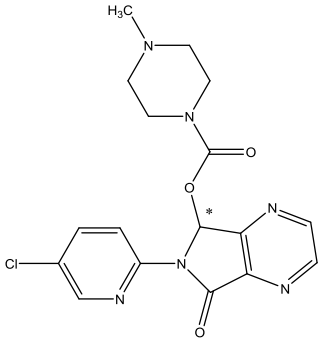
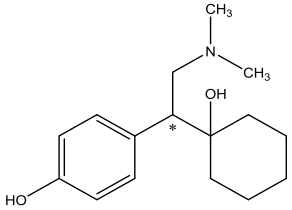
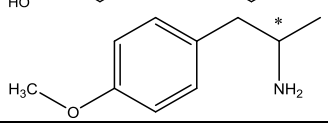
**Table 3-5** Validation parameters - retention time, relative retention time, linearity range, correlation coefficient obtained from calibration curve and instrumental and method limits of detection and instrumental and method limits of quantification (WW means wastewater).

| Compound | $R_t$ (min) | Rel. $R_t$ | Sample diluent | | | | Influent WW | |
|---|---|---|---|---|---|---|---|---|
| | | | Linearity range (µg/L) | $R^2$ | $IDL_{S/N}$ (µg/L) | $IQL_{S/N}$ (µg/L) | MDL (µg/L) | MQL (µg/L) |
| Cocaine | 15.7 ±0.4 | 0.3 | 0.010-1000 | 0.9997 | 0.01 | 0.05 | 0.0001 | 0.0003 |
| Benzoylecgonine | 3.1 ±0.0 | 0.0 | 0.005-1000 | 0.9992 | 0.01 | 0.02 | 0.0001 | 0.0001 |
| Cocaethylene | 16.0 ±0.7 | 0.3 | 0.100-1000 | 0.9996 | 0.10 | 0.25 | 0.0005 | 0.0013 |
| *R*-(-)-Amphetamine | 15.5 ±0.3 | 0.1 | 0.125-500 | 0.9987 | 0.12 | 0.50 | 0.0008 | 0.0029 |
| *S*-(+)-Amphetamine | 22.6 ±0.4 | 0.2 | 0.125-500 | 0.9988 | 0.12 | 0.50 | 0.0008 | 0.0029 |
| *R*-(-)-Methamphetamine | 14.5 ±0.4 | 0.3 | 0.050-500 | 0.9989 | 0.05 | 0.12 | 0.0003 | 0.0006 |
| *S*-(+)-Methamphetamine | 16.5 ±0.4 | 0.3 | 0.050-500 | 0.9994 | 0.05 | 0.12 | 0.0003 | 0.0007 |
| E1-Mephedrone | 16.5 ±0.4 | 0.3 | 0.250-500 | 0.9990 | 0.25 | 0.50 | 0.0013 | 0.0026 |
| E2-Mephedrone | 21.0 ±0.5 | 0.2 | 0.250-500 | 0.9993 | 0.25 | 0.50 | 0.0007 | 0.0026 |
| *R*-(-)-MDA | 28.1 ±0.5 | 0.2 | 0.500-500 | 0.9991 | 0.50 | 2.50 | 0.0028 | 0.0140 |
| *S*-(+)-MDA | 47.4 ±0.8 | 0.4 | 0.500-500 | 0.9980 | 0.50 | 2.50 | 0.0025 | 0.0124 |
| *R*-(-)-MDMA | 21.9 ±0.5 | 0.2 | 0.050-500 | 0.9992 | 0.05 | 0.25 | 0.0003 | 0.0014 |
| *S*-(+)-MDMA | 32.9 ±0.5 | 0.1 | 0.050-500 | 0.9994 | 0.05 | 0.25 | 0.0003 | 0.0013 |
| E1-MDEA | 19.0 ±0.5 | 1.8 | 0.125-500 | 0.9994 | 0.12 | 0.25 | 0.0006 | 0.0013 |
| E2-MDEA | 21.0 ±0.5 | 0.2 | 0.125-500 | 0.9995 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| Heroin | 22.5 ±0.4 | 0.5 | 1.000-1000 | 0.9946 | 1.00 | 5.00 | 0.0062 | 0.0312 |
| *O*-6-monoacetylmorphine | 24.1 ±0.7 | 1.1 | 0.250-1000 | 0.9987 | 0.25 | 1.00 | 0.0011 | 0.0044 |
| Morphine | 17.4 ±0.8 | 0.5 | 0.250-1000 | 0.9955 | 0.25 | 0.50 | 0.0012 | 0.0025 |
| Morphine-3β-*D*-glucuronide | 3.3 ±0.0 | 6.5 | 0.500-1000 | 0.9983 | 0.50 | 5.00 | 0.0023 | 0.0228 |
| Ketamine | 11.6 ±0.2 | 0.3 | 0.100-1000 | 0.9994 | 0.10 | 0.25 | 0.0005 | 0.0012 |
| Benzylpiperazine | 21.3 ±0.4 | 1.6 | 0.500-1000 | 0.9957 | 0.50 | 1.00 | 0.0024 | 0.0048 |
| Temazepam | 5.2 ±0.2 | 1.2 | 0.250-1000 | 0.9972 | 0.25 | 0.50 | 0.0011 | 0.0022 |
| Diazepam | 6.0 ±0.3 | 0.6 | 0.250-1000 | 0.9974 | 0.25 | 0.50 | 0.0012 | 0.0024 |
| Nordiazepam | 8.9 ±0.2 | 0.5 | 0.250-1000 | 0.9985 | 0.25 | 0.50 | 0.0012 | 0.0024 |
| Nitrazepam | 7.3 ±0.0 | 1.4 | 0.250-1000 | 0.9984 | 0.25 | 0.50 | 0.0014 | 0.0027 |
| Oxazepam | 7.0 ±0.2 | 4.8 | 0.500-1000 | 0.9971 | 0.50 | 1.00 | 0.0025 | 0.0049 |
| 7-amino-nitrazepam | 5.3 ±0.1 | 0.6 | 0.250-1000 | 0.9923 | 0.25 | 0.50 | 0.0015 | 0.0030 |
| Lorazepam | 6.8 ±0.1 | 1.4 | 1.000-800 | 0.9900 | 1.00 | 5.00 | 0.0051 | 0.0256 |
| Anhydroecgonine methyl ester | 12.4 ±0.2 | 0.5 | 0.500-1000 | 0.9971 | 0.50 | 1.00 | 0.0028 | 0.0056 |
| E1-HMA | 17.7 ±0.4 | 0.4 | 2.500-500 | 0.9900 | 2.50 | 5.00 | 0.0118 | 0.0236 |
| E2-HMA | 34.3 ±0.5 | 0.8 | 2.500-500 | 0.9903 | 2.50 | 5.00 | 0.0113 | 0.0225 |
| E1-HMMA | 15.9 ±0.4 | 2.5 | 0.250-500 | 0.9982 | 0.25 | 0.50 | 0.0014 | 0.0028 |
| E2-HMMA | 18.6 ±0.5 | 2.5 | 0.250-500 | 0.9974 | 0.25 | 0.50 | 0.0011 | 0.0022 |
| DHMA | 12.5 ±0.2 | 4.1 | 1.000-1000 | 0.9959 | 1.00 | 5.00 | 0.0045 | 0.0226 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Caffeine | 6.1 ±0.0 | 0.8 | 0.250-1000 | 0.9981 | 0.25 | 0.50 | 0.0047 | 0.0259 |
| 1,7-Dimethylxanthine | 6.4 ±0.1 | 0.8 | 1.000-1000 | 0.9983 | 1.00 | 5.00 | 0.0048 | 0.0241 |
| Nicotine | 12.5 ±0.1 | 2.6 | 0.250-1000 | 0.9964 | 0.25 | 0.50 | 0.0013 | 0.0025 |
| Cotinine | 3.4 ±0.0 | 0.5 | 0.010-1000 | 0.9988 | 0.01 | 0.02 | 0.0001 | 0.0001 |
| Creatinine | 3.0 ±0.0 | 2.0 | 1.000-1000 | 0.9943 | 1.00 | 5.00 | 0.0053 | 0.0265 |
| Codeine | 15.8 ±0.4 | 0.3 | 0.500-1000 | 0.9980 | 0.50 | 1.00 | 0.0024 | 0.0048 |
| Oxycodone | 16.1 ±0.7 | 0.3 | 0.250-1000 | 0.9977 | 0.25 | 1.00 | 0.0014 | 0.0054 |
| Noroxycodone | 20.7 ±0.3 | 0.6 | 1.000-1000 | 0.9991 | 1.00 | 5.00 | 0.0057 | 0.0285 |
| Hydrocodone | 19.2 ±1.1 | 0.4 | 1.000-1000 | 0.9987 | 1.00 | 5.00 | 0.0052 | 0.0259 |
| Oxymorphone | 18.7 ±0.5 | 0.3 | 1.000-1000 | 0.9976 | 1.00 | 5.00 | 0.0056 | 0.0278 |
| Dihydrocodeine | 14.0 ±0.5 | 0.6 | 0.500-1.000 | 0.9985 | 0.50 | 1.00 | 0.0026 | 0.0051 |
| Methadone | 21.3 ±1.3 | 0.3 | 0.250-1000 | 0.9992 | 0.25 | 0.50 | 0.0012 | 0.0025 |
| EDDP | 19.7 ±0.5 | 0.2 | 0.025-1000 | 0.9993 | 0.02 | 0.10 | 0.0001 | 0.0005 |
| E1-Venlafaxine | 12.5 ±0.5 | 0.6 | 0.125-500 | 0.9980 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| E2-Venlafaxine | 15.6 ±0.5 | 2.9 | 0.125-500 | 0.9971 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| Vardenafil | 24.7 ±1.3 | 2.8 | 1.000-1000 | 0.9911 | 1.00 | 5.00 | 0.0045 | 0.0223 |
| E1-Norephedrine | 13.6 ±0.3 | 0.4 | 0.125-500 | 0.9981 | 0.12 | 0.25 | 0.0006 | 0.0011 |
| E2-Norephedrine | 15.1 ±0.4 | 2.2 | 0.125-500 | 0.9983 | 0.12 | 0.25 | 0.0006 | 0.0012 |
| E1-PMA | 21.3 ±0.5 | 0.5 | 0.125-500 | 0.9964 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| E2-PMA | 36. 8 ±0.4 | 1.4 | 0.125-500 | 0.9994 | 0.12 | 0.25 | 0.0006 | 0.0011 |
| Normorphine | 20.0 ±0.6 | 0.8 | 1.000-800 | 0.9905 | 1.00 | 5.00 | 0.0055 | 0.0276 |
| Dihydromorphine | 15.1 ±0.5 | 0.5 | 1.000-800 | 0.9915 | 1.00 | 5.00 | 0.0056 | 0.0282 |
| D1-Tramadol | 12.6 ±0.4 | 0.6 | 0.500-500 | 0.9985 | 0.50 | 1.00 | 0.0024 | 0.0047 |
| D2-Tramadol | 13.7 ±0.5 | 0.7 | 0.500-500 | 0.9989 | 0.50 | 1.00 | 0.0029 | 0.0059 |
| *O*-Demethyltramadol | 13.5 ±0.4 | 0.8 | 0.500-1000 | 0.9921 | 0.50 | 1.00 | 0.0027 | 0.0053 |
| Zolpidem | 15.1 ±0.6 | 2.3 | 0.025-1000 | 0.9924 | 0.02 | 1.00 | 0.0001 | 0.0047 |
| Amitriptyline | 55.3±3.1 | 2.9 | 5.000-1000 | 0.9950 | 5.00 | 10.00 | 0.0294 | 0.0588 |
| Norketamine | 8.5 ±0.3 | 0.6 | 0.500-1000 | 0.9986 | 0.50 | 1.00 | 0.0024 | 0.0048 |
| Sildenafil | 17.7 ±1.0 | 3.8 | 1.000-1000 | 0.9911 | 1.00 | 5.00 | 0.0047 | 0.0237 |
| (+)-Ephedrine | 12.3 ±0.3 | 0.6 | 1.000-500 | 0.9974 | 1.00 | 5.00 | 0.0059 | 0.0295 |
| (-)-Ephedrine and (-)-Ψephedrine | 13.4 ±0. | 0.5 | 0.500-1000 | 0.9975 | 0.50 | 1.00 | 0.0024 | 0.0048 |
| (+)-Ψephedrine | 32.94 ±0.8 | 1.9 | 1.000-500 | 0.9903 | 1.00 | 5.00 | 0.0056 | 0.0280 |
| Desmethylvenlafaxine-E1 | 15.8 ±0.4 | 0.7 | 5.000-500 | 0.9941 | 5.000 | 10.000 | 0.0249 | 0.0497 |
| Desmethylvenlafaxine-E2 | 17.2 ±0.4 | 0.6 | 5.000-500 | 0.9973 | 5.000 | 10.000 | 0.0275 | 0.0550 |
| E1-Zopiclone | 32.7 ±0.3 | 4.6 | 10.000-500 | 0.9903 | 10.000 | 50.000 | 0.0285 | 0.3125 |
| E2-Zopiclone | 59.8 ±0.4 | 5.2 | 10.000-500 | 0.9909 | 10.000 | 50.000 | 0.0326 | 0.3208 |
| *S*-(+)-Fluoxetine | 43.2 ±1.8 | 3.1 | 10.000-500 | 0.9915 | 10.000 | 50.000 | 0.0533 | 0.2664 |
| *R*-(-)-Fluoxetine | 57.2 ±2.1 | 3.3 | 10.000-500 | 0.9907 | 10.000 | 50.000 | 0.0517 | 0.2588 |
| E1-Norfluoxetine | 81.3 ±6.0 | 14.4 | 10.000-500 | 0.9916 | 10.000 | 50.000 | 0.0589 | 0.2945 |
| E2-Norfluoxetine | 87.8 ±3.5 | 12.9 | 10.000-500 | 0.9921 | 10.000 | 50.000 | 0.0612 | 0.3061 |

**Table 3-6** Validation parameters - enantiomeric fraction (EF) and enantiomeric resolution (Rs) of compounds, which enantiomers were separated under studied conditions.

| Analyte | Structure | Rs | EF (n=9) | | |
|---|---|---|---|---|---|
| | | | 10 µg/L | 100 µg/L | 1000 µg/L |
| Amphetamine |  | 1.2±0.1 | 0.47±0.01 | 0.49±0.02 | 0.48±0.01 |
| Methamphetamine |  | 1.0±0.0 | 0.50±0.00 | 0.49±0.00 | 0.49±0.00 |
| Mephedrone |  | 1.4±0.1 | 0.50±0.01 | 0.50±0.00 | 0.48±0.01 |
| MDA |  | 1.8±0.2 | 0.47±0.01 | 0.48±0.01 | 0.50±0.00 |
| MDMA |  | 1.2±0.1 | 0.51±0.00 | 0.50±0.00 | 0.51±0.00 |
| MDEA |  | 0.8±0.3 | 0.50±0.01 | 0.51±0.01 | 0.50±0.01 |
| HMA |  | 2.7±0.3 | 0.54±0.08 | 0.47±0.00 | 0.49±0.05 |
| HMMA |  | 0.8±0.1 | 0.48±0.01 | 0.43±0.01 | 0.40±0.00 |
| Tramadol |  | 0.9±0.0 | 0.46±0.01 | 0.46±0.02 | 0.49±0.03 |
| Fluoxetine |  | 0.6±0.2 | 0.51±0.03 | 0.50±0.02 | 0.51±0.04 |

| | | | | | |
|---|---|---|---|---|---|
| Norfluoxetine |  | 1.9±0.10 | 0.50±0.08 | 0.47±0.05 | 0.50±0.07 |
| Venlafaxine |  | 1.0±0.1 | 0.50±0.04 | 0.49±0.01 | 0.50±0.01 |
| (+)-Ephedrine |  | 0.9±0.1 | 0.40±0.03 | 0.50±0.1 | 0.49±0.16 |
| (+)-Ψephedrine |  | 2.2±0.2 | 0.52±0.01 | 0.45±0.03 | 0.42±0.02 |
| Norephedrine |  | 0.9±0.1 | 0.50±0.04 | 0.46±0.01 | 0.47±0.00 |
| Zopiclone |  | 3.1±0.2 | 0.43±0.02 | 0.47±0.01 | 0.48±0.02 |
| Desmethylvenlafaxine |  | 0.9±0.1 | 0.42±0.04 | 0.43±0.03 | 0.40±0.03 |
| PMA |  | 2.7±0.2 | 0.48±0.02 | 0.47±0.01 | 0.41±0.00 |

The following analytes: MDEA, HMMA, tramadol, fluoxetine, ephedrine, norephedrine and desmethylvenlafaxine showed lower enantiomeric resolution and therefore results for single enantiomers of these compounds should be considered on a semi-quantitative basis.

Enantiomeric fractions for those analytes which were injected as racemates, were on average 0.49 and were reproducible across different concentration ranges (Table 3-6).

The instrumental limits of detection and quantification ranged from 0.005 to 10 µg $L^{-1}$ and from 0.050 to 50 µg $L^{-1}$, respectively (Table 3-5). The method limits of detection and quantification ranged from 0.03 to 61 ng $L^{-1}$ and from 0.130 to 320.870 ng $L^{-1}$ (Table 3-5). The instrumental and method precision was on average <5% and <10% respectively (Tables 3-7 and S5).

Ion suppression studies showed how the presence of the internal standard deuterates compensated the ion suppression in the matrix, even for those compounds that had not its corresponding deuterated analogue. (Table S6).

### 3.4.4 Analysis of wastewater samples

The developed and validated method was applied in a one-week monitoring campaign of a wastewater treatment plant serving a large city in the UK. The results are provided in Table 3-8.

Most target drugs were found at quantifiable concentrations in analysed samples. The results for several drugs such as cocaine and MDMA and their metabolites show a clear trend of increased concentration during weekends. Other target drugs showed constant concentrations across the sampling week. These are for example: morphine, ketamine, benzylpiperazine, dihydrocodeine, methadone, amphetamine and methamphetamine. These results will be used in further study to estimate drug use via wastewater-based epidemiology. It is worth noting that despite suspected high usage of zopiclone, fluoxetine and norfluoxetine, these drugs were not detected in analysed wastewater samples. This is probably because of relatively high MDL values for zopiclone, fluoxetine and its metabolite norfluoxetine in the developed method. Amphetamine and MDMA were found enriched with *R*-(-)-enantiomers, probably due to their stereoselective metabolism favouring *S*-(+)-enantiomers. MDA was either enriched with *R*-(-)- or *S*-(+)-enantiomer indicating that its presence might be due to either abuse of racemic MDA (excess of *R*-(-)-enantiomer

should be observed if administered as racemate) or abuse of racemic MDMA (excess of *S*-(+)-enantiomer should be observed).

**Table 3-7** Validation parameters - method precision

| Analytes | Intra-day RSD% (n=4) | | | | | | | | | Inter-day RSD% (n=3) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 ng/L** D 1* | 25 ng/L D 2 | 25 ng/L D 3 | 250 ng/L D 1 | 250 ng/L D 2 | 250 ng/L D 3 | 2500 ng/L D 1 | 2500 ng/L D 2 | 2500 ng/L D 3 | 25 ng/L | 250 ng/L | 2500 ng/L |
| Cocaine | 6.5 | 2.7 | 5.2 | 0.5 | 4.8 | 3.4 | 4.7 | 1.9 | 1.5 | 4.8 | 2.9 | 2.7 |
| Benzoylecgonine | 2.3 | 7.5 | 10.2 | 5.3 | 4.6 | 6.6 | 14.4 | 2.5 | 4.9 | 6.6 | 5.5 | 7.3 |
| Cocaethylene | 3.5 | 5.1 | 5.4 | 6.4 | 3.8 | 3.8 | 6.1 | 2.0 | 4.3 | 4.7 | 4.7 | 4.2 |
| *R*-(-)-Amphetamine | 3.3 | 2.5 | 4.6 | 5.2 | 14.7 | 10.8 | 6.2 | 3.9 | 6.2 | 3.5 | 10.2 | 5.4 |
| *S*-(+)-Amphetamine | 3.1 | 4.3 | 12.6 | 1.4 | 6.5 | 4.7 | 3.8 | 7.0 | 7.3 | 6.7 | 4.2 | 6.0 |
| *R*-(-)-Methamphetamine | 8.9 | 6.7 | 9.3 | 3.4 | 7.0 | 8.3 | 4.8 | 5.2 | 5.4 | 8.3 | 6.2 | 5.1 |
| *S*-(+)-Methamphetamine | 6.8 | 3.6 | 15.4 | 1.2 | 5.5 | 4.0 | 2.7 | 2.9 | 4.2 | 8.6 | 3.6 | 3.3 |
| E1-Mephedrone | 9.8 | 13.7 | 14.1 | 3.6 | 6.8 | 14.6 | 3.7 | 10.0 | 5.6 | 12.5 | 8.3 | 6.4 |
| E2-Mephedrone | 10.7 | 12.0 | 4.6 | 5.2 | 12.9 | 8.4 | 9.2 | 3.7 | 2.8 | 9.1 | 8.8 | 5.2 |
| *R*-(-)-MDA | 1.7 | 6.6 | 9.7 | 3.0 | 3.4 | 5.7 | 0.1 | 7.7 | 1.1 | 6.0 | 4.0 | 3.0 |
| *S*-(+)-MDA | 4.4 | 3.8 | 7.8 | 2.6 | 6.7 | 5.3 | 7.2 | 3.7 | 4.5 | 5.3 | 4.9 | 5.1 |
| *R*-(-)-MDMA | 7.0 | 1.8 | 4.0 | 5.8 | 4.6 | 3.9 | 3.4 | 1.5 | 6.5 | 4.3 | 4.8 | 3.8 |
| *S*-(+)-MDMA | 1.0 | 1.9 | 6.9 | 0.6 | 3.1 | 2.9 | 1.2 | 2.8 | 0.7 | 3.3 | 2.2 | 1.6 |
| E1-MDEA | 6.9 | 6.2 | 3.0 | 5.1 | 8.5 | 7.8 | 4.7 | 2.2 | 4.3 | 5.4 | 7.1 | 3.7 |
| E2-MDEA | 6.0 | 6.3 | 2.8 | 1.4 | 9.2 | 4.9 | 8.3 | 1.4 | 1.7 | 5.0 | 5.2 | 3.8 |
| Heroin | 17.3 | 12.0 | 1.1 | 4.4 | 6.8 | 6.4 | 10.5 | 5.2 | 12.2 | 10.1 | 5.9 | 9.3 |
| *O*-6-monoacetylmorphine | 3.3 | 6.9 | 12.1 | 4.2 | 6.6 | 5.7 | 5.8 | 3.8 | 6.3 | 7.4 | 5.5 | 5.3 |
| Morphine | 17.8 | 0.8 | 0.8 | 8.8 | 4.5 | 6.7 | 6.7 | 7.2 | 14.2 | 6.5 | 6.7 | 9.4 |
| Morphine-3β-*D*-glucuronide | 18.2 | 3.7 | 23.2 | 27.1 | 10.4 | 4.8 | 18.6 | 19.4 | 4.2 | 15.0 | 14.1 | 14.1 |
| Ketamine | 8.3 | 2.2 | 3.9 | 2.0 | 5.2 | 3.9 | 1.9 | 1.6 | 2.0 | 4.8 | 3.7 | 1.8 |
| Benzylpiperazine | 4.4 | 1.0 | 9.4 | 4.3 | 7.1 | 5.2 | 1.9 | 4.8 | 2.1 | 5.0 | 5.5 | 2.9 |
| Temazepam | 25.9 | 16.4 | 5.5 | 6.7 | 8.7 | 8.5 | 4.1 | 3.8 | 2.7 | 15.9 | 8.0 | 3.5 |
| Diazepam | 2.1 | 5.4 | 5.8 | 5.0 | 9.5 | 8.4 | 1.8 | 3.9 | 2.7 | 4.4 | 7.6 | 2.8 |
| Nordiazepam | 3.1 | 19.8 | 7.0 | 4.7 | 5.5 | 5.6 | 15.7 | 5.1 | 6.1 | 9.9 | 5.3 | 9.0 |
| Nitrazepam | 9.0 | 1.2 | 18.7 | 9.2 | 4.5 | 5.2 | 6.2 | 5.1 | 1.9 | 9.6 | 6.3 | 4.4 |
| Oxazepam | 13.7 | 10.0 | 10.7 | 3.9 | 8.3 | 5.6 | 3.4 | 5.3 | 7.9 | 11.4 | 5.9 | 5.5 |
| 7-amino-nitrazepam | 0.0 | 4.5 | 5.0 | 3.4 | 5.8 | 2.0 | 0.0 | 2.1 | 5.2 | 3.2 | 3.7 | 2.4 |
| Lorazepam | 4.4 | 10.6 | 6.7 | 9.9 | 3.9 | 2.4 | 3.9 | 6.8 | 3.3 | 7.2 | 5.4 | 4.7 |
| Anhydroecgonine methyl ester | 5.3 | 9.3 | 3.6 | 1.4 | 5.6 | 5.7 | 3.0 | 3.2 | 1.0 | 6.1 | 4.2 | 2.4 |
| E1-HMA | 4.4 | 5.1 | 1.6 | 7.6 | 1.1 | 4.4 | 6.4 | 6.0 | 5.9 | 3.7 | 4.4 | 6.1 |
| E2-HMA | 5.2 | 4.8 | 12.6 | 3.8 | 2.0 | 5.0 | 7.0 | 6.5 | 6.0 | 7.5 | 3.6 | 6.5 |
| E1-HMMA | 7.4 | 7.6 | 7.5 | 2.8 | 3.8 | 6.0 | 4.1 | 2.7 | 0.3 | 7.5 | 4.2 | 2.4 |
| E2-HMMA | 4.7 | 6.4 | 3.6 | 2.1 | 2.1 | 6.2 | 2.9 | 3.1 | 3.6 | 4.9 | 3.5 | 3.2 |
| DHMA | 8.9 | 9.1 | 1.2 | 6.1 | 2.5 | 9.0 | 3.2 | 6.9 | 4.6 | 6.4 | 5.9 | 4.9 |
| Caffeine | 2.2 | 5.6 | 2.2 | 5.1 | 7.3 | 3.8 | 9.5 | 0.9 | 1.9 | 3.3 | 5.4 | 4.1 |
| 1,7-Dimethylxanthine | 6.0 | 4.0 | 5.7 | 4.5 | 0.0 | 4.7 | 2.7 | 3.5 | 5.8 | 5.2 | 3.1 | 4.0 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (-)-Nicotine | 3.7 | 4.0 | 8.5 | 6.6 | 1.8 | 4.0 | 1.6 | 5.5 | 5.4 | 5.4 | 4.1 | 4.1 |
| Cotinine | 3.0 | 8.1 | 6.8 | 4.4 | 4.0 | 4.8 | 3.2 | 6.1 | 4.7 | 6.0 | 4.4 | 4.7 |
| Creatinine | 18.3 | 1.0 | 9.7 | 2.7 | 14.9 | 8.8 | 0.7 | 7.6 | 4.4 | 9.6 | 8.8 | 4.2 |
| Codeine | 2.9 | 4.5 | 4.9 | 2.1 | 5.7 | 7.8 | 6.4 | 2.7 | 9.0 | 4.1 | 5.2 | 6.0 |
| Oxycodone | 10.5 | 4.2 | 16.8 | 2.3 | 4.4 | 8.6 | 3.3 | 7.0 | 7.6 | 10.5 | 5.1 | 6.0 |
| Noroxycodone | 19.3 | 9.2 | 14.1 | 2.6 | 9.3 | 7.4 | 4.9 | 8.3 | 5.4 | 14.2 | 6.4 | 6.2 |
| Hydrocodone | 4.7 | 1.1 | 1.9 | 3.8 | 9.2 | 7.1 | 1.4 | 5.5 | 7.8 | 2.6 | 6.7 | 4.9 |
| Oxymorphone | 5.3 | 2.1 | 6.7 | 3.7 | 0.6 | 3.5 | 4.9 | 3.5 | 6.7 | 4.7 | 2.6 | 5.0 |
| Dihydrocodeine | 0.3 | 7.6 | 4.7 | 1.0 | 7.0 | 1.7 | 5.6 | 3.9 | 3.7 | 4.2 | 3.2 | 4.4 |
| Methadone | 7.8 | 0.0 | 7.3 | 2.1 | 4.4 | 6.5 | 5.9 | 2.8 | 5.7 | 5.0 | 4.4 | 4.8 |
| EDDP | 3.1 | 5.3 | 6.2 | 3.9 | 9.4 | 5.3 | 5.1 | 1.1 | 2.9 | 4.9 | 6.2 | 3.0 |
| E1-Venlafaxine | 9.1 | 1.5 | 5.7 | 5.5 | 5.2 | 7.5 | 5.3 | 7.1 | 5.6 | 5.4 | 6.1 | 6.0 |
| E2-Venlafaxine | 0.0 | 4.8 | 3.1 | 4.9 | 1.4 | 7.6 | 1.5 | 4.0 | 5.2 | 2.6 | 4.6 | 3.6 |
| Vardenafil | 9.4 | 11.0 | 10.6 | 5.6 | 9.2 | 13.0 | 14.6 | 9.0 | 5.2 | 10.3 | 9.3 | 9.6 |
| E1-Norephedrine | 7.3 | 3.8 | 1.3 | 2.8 | 3.0 | 7.3 | 4.4 | 3.0 | 7.4 | 4.1 | 4.3 | 5.0 |
| E2-Norephedrine | 5.7 | 4.6 | 6.3 | 3.1 | 3.9 | 6.1 | 2.2 | 2.1 | 3.5 | 5.5 | 4.3 | 2.6 |
| E1-PMA | 7.7 | 4.8 | 8.3 | 1.4 | 4.4 | 3.7 | 3.8 | 4.3 | 5.3 | 6.9 | 3.2 | 4.5 |
| E2-PMA | 6.2 | 8.8 | 11.6 | 7.8 | 4.6 | 6.6 | 1.7 | 3.9 | 2.9 | 8.9 | 6.3 | 2.8 |
| Normorphine | 11.4 | 2.9 | 4.6 | 2.7 | 12.6 | 5.5 | 12.0 | 3.6 | 7.8 | 6.3 | 6.9 | 7.8 |
| Dihydromorphine | 1.5 | 13.4 | 4.1 | 10.1 | 14.9 | 1.4 | 9.8 | 2.6 | 11.3 | 6.3 | 8.8 | 7.9 |
| D1-Tramadol | 4.9 | 7.0 | 6.6 | 6.1 | 5.7 | 3.9 | 6.5 | 1.7 | 0.5 | 6.2 | 5.3 | 2.9 |
| D2-Tramadol | 6.2 | 9.7 | 6.1 | 4.2 | 3.2 | 4.0 | 2.5 | 3.7 | 2.5 | 7.3 | 3.8 | 2.9 |
| *O*-Desmethyltramadol | 4.8 | 8.7 | 0.0 | 15.7 | 16.0 | 4.6 | 12.2 | 16.2 | 11.5 | 4.5 | 12.1 | 13.3 |
| Zolpidem | 18.2 | 7.0 | 0.6 | 5.5 | 3.4 | 4.8 | 0.6 | 9.1 | 6.9 | 8.6 | 4.5 | 5.5 |
| Amitriptyline | 8.0 | 7.3 | 1.1 | 10.9 | 7.3 | 8.7 | 2.7 | 6.7 | 0.1 | 5.5 | 9.0 | 3.1 |
| Norketamine | 9.6 | 7.6 | 11.2 | 7.7 | 8.0 | 8.3 | 6.5 | 5.8 | 3.6 | 9.5 | 8.0 | 5.3 |
| Sildenafil | 20.1 | 5.9 | 20.8 | 1.2 | 13.3 | 10.1 | 5.6 | 3.5 | 6.8 | 15.6 | 8.2 | 5.3 |
| (+)-Ephedrine | 5.3 | 16.5 | 9.8 | 5.0 | 4.5 | 6.6 | 7.2 | 2.8 | 3.3 | 10.5 | 5.4 | 4.4 |
| (-)-Ephedrine and (-)-Ψephedrine | 8.3 | 14.8 | 5.2 | 1.8 | 0.8 | 5.4 | 5.7 | 1.0 | 3.3 | 9.4 | 2.7 | 3.3 |
| (+)-Ψephedrine | 2.8 | 2.5 | 6.2 | 5.8 | 1.3 | 9.4 | 2.9 | 2.0 | 1.7 | 3.8 | 5.5 | 2.2 |
| Desmethylvenlafaxine-E1 | 8.7 | 7.4 | 2.3 | 8.4 | 3.7 | 9.5 | 2.7 | 5.0 | 3.7 | 6.2 | 7.2 | 3.8 |
| Desmethylvenlafaxine-E2 | 6.4 | 8.7 | 7.4 | 3.8 | 2.8 | 5.3 | 2.3 | 4.9 | 8.2 | 7.5 | 4.0 | 5.1 |
| E1-Zopiclone | 20.0 | 17.8 | 19.5 | 14.5 | 13.2 | 19.2 | 12.6 | 7.9 | 5.6 | 19.1 | 15.6 | 8.7 |
| E2-Zopiclone | 18.7 | 18.2 | 20.4 | 17.6 | 14.8 | 6.9 | 11.4 | 5.8 | 9.8 | 19.2 | 13.1 | 9.0 |
| *S*-(+)-Fluoxetine | 19.3 | 14.2 | 1.1 | 12.9 | 18.2 | 14.0 | 3.5 | 5.4 | 2.9 | 11.5 | 15.0 | 4.0 |
| *R*-(-)-Fluoxetine | 19.2 | 2.7 | 20.7 | 6.2 | 3.8 | 0.5 | 0.3 | 2.7 | 14.5 | 14.2 | 3.5 | 5.8 |
| E1-Norfluoxetine | 17.6 | 15.1 | 9.7 | 6.6 | 3.8 | 9.6 | 2.5 | 7.9 | 13.5 | 14.1 | 6.7 | 8.0 |
| E2-Norfluoxetine | 1.9 | 6.8 | 10.7 | 20.3 | 10.5 | 3.4 | 0.7 | 10.7 | 7.3 | 6.5 | 11.4 | 6.2 |

*-D indicates day

**- the following concentrations were used: 10, 100 and 1000 ng $L^{-1}$ in the case of compounds that were not enantioseparated

As MDA is a minor and not exclusive metabolite of MDMA, other metabolites (HMMA, HMA, and DHMA) were targeted for the first time in wastewater. The trend observed for HMMA in terms of concentration was similar to the parent drug MDMA, whilst for HMA and DHMA the trends were not "superimposable" to that one of MDMA. Among the metabolites of MDMA investigated, this was the first time that the enantiomeric profiling of HMA and HMMA was studied in wastewater. In the developed method, the enantiomers of DHMA were not separated so evaluation of its enantiomeric profiling was not possible. Significant changes in enantiomeric fractions (between 0.40 and 0.58) were noticed in the case of HMMA suggesting enantioselective metabolism. The enantiomeric profiling of PMA was not undertaken as PMA was not detected in wastewater. Even though PMA is a minor metabolite of PMMA, as reported by Lin et al.2007, most PMMA is excreted unchanged in the urine. So, PMA could be a suitable biomarker only for PMA intake [37].

Temporal changes in mephedrone concentrations were observed with noticeable increase of mephedrone levels during weekends. This is the first time mephedrone was detected and quantified in wastewater in the UK. Mephedrone was also found to be enriched with E1 enantiomer, which suggests enantioselective metabolism in humans. Further work is needed to support the above hypothesis.

Moreover, the method was applied for investigating the in-sewer stability of the targeted biomarkers in a pressurized sewer under anaerobic conditions ( see Figure 3-1). As WBE relies on the quantification of a DTR and a DTR may undergo degradation during the transport in the sewer until the collection of the wastewater sample, in-sewer stability studies are required. Indeed, they can evaluate any alteration in its concentration avoiding any under- or over-estimations of the targeted drug in WBE. This study was performed for the first time at enantiomeric level. Nearly 44% of the compounds were highly stable (changes in concentrations were within 20%), 10% had medium stability (variations were between 20 and 40%), 3% had low stability (alterations >40%). 43% of the compounds were <MDL as they were not present in the wastewater collected at the inlet of the pipe ( Table 3-9).

**Table 3-8** Concentrations of targeted compounds in wastewater samples during one week monitoring campaign.

| | Concentration [ng $L^{-1}$] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
| Cocaine | 403 ± 28 | 449 ± 60 | 420 ± 13 | 397 ± 28 | 452 ± 22 | 694 ± 23 | 634 ± 23 |
| Benzoylecgonine | 997 ± 150 | 754 ± 90 | 788 ± 26 | 864 ± 112 | 950 ± 43 | 1604 ± 129 | 1537 ± 95 |
| Cocaethylene | 4 ± 0 | 2 ± 0 | 2 ± 1 | 2 ± 0 | 4 ± 0 | 10 ± 1 | 9 ± 1 |
| *R*-(-)-Amphetamine | 241 ± 62 | 169 ± 8 | 207 ± 39 | 202 ± 14 | 204 ± 10 | 224 ± 17 | 192 ± 11 |
| *S*-(+)-Amphetamine | 171 ± 12 | 122 ± 7 | 154 ± 7 | 152 ± 14 | 140 ± 28 | 170 ± 17 | 147 ± 3 |
| *R*-(-)-Methamphetamine | 6 ± 1 | 3 ± 11 | 6 ± 1 | 6 ± 2 | 6 ± 1 | 5 ± 1 | 4 ± 1 |
| *S*-(+)-Methamphetamine | 2 ± 2 | 3 ± 5 | 6 ± 1 | 6 ± 4 | 3 ± 1 | 3 ± 2 | 4 ± 1 |
| E1-Mephedrone | 42 ± 7 | 18 ± 6 | 32 ± 10 | 18 ± 3 | 22 ± 7 | 67 ± 15 | 53 ± 11 |
| E2-Mephedrone | 29 ± 2 | 14 ± 5 | 28 ± 3 | 14 ± 6 | 18 ± 5 | 47 ± 6 | 44 ± 5 |
| *R*-(-)-MDA | 7 ± 7 | 3 ± 4 | 10 ± 11 | N.D. | N.D. | 4 ± 3 | 7 ± 2 |
| *S*-(+)-MDA | N.D. | N.D. | 2 ± 4 | 3 ± 4 | 4 ± 8 | 13 ± 5 | 14 ± 7 |
| *R*-(-)-MDMA | 109 ± 7 | 68 ± 5 | 45 ± 3 | 34 ± 2 | 45 ± 4 | 133 ± 9 | 186 ± 10 |
| *S*-(+)-MDMA | 43 ± 4 | 26 ± 2 | 23 ± 2 | 19 ± 2 | 32 ± 1 | 84 ± 4 | 110 ± 6 |
| E1-MDEA | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E2-MDEA | 1 ± 0 | 1 ± 1 | N.D. | N.D. | 8 ± 16 | N.D. | 1 ± 1 |
| Heroin | N.D. | 26 ± 52 | 112 ± 223 | 68 ± 78 | 50 ± 64 | 147 ± 24 | 16 ± 32 |
| *O*-6-monoacetylmorphine | 7 ± 4 | 2 ± 2 | 5 ± 4 | 2 ± 2 | 4 ± 4 | 7 ± 5 | 2 ± 2 |
| Morphine | 653 ± 29 | 643 ± 65 | 713 ± 40 | 514 ± 18 | 640 ± 27 | 591 ± 63 | 595 ± 46 |
| Morphine-3β-*D*-glucuronide | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Ketamine | 274 ± 17 | 235 ± 9 | 284 ± 22 | 250 ± 14 | 254 ± 8 | 287 ± 23 | 281 ± 14 |
| Benzylpiperazine | 9 ± 7 | 65 ± 6 | 9 ± 3 | 9 ± 2 | 7 ± 1 | 7 ± 3 | 8 ± 3 |
| Temazepam | 269 ± 78 | 320 ± 116 | 408 ± 55 | 233 ± 125 | 224 ± 133 | 256 ± 134 | 255 ± 119 |
| Diazepam | 3 ± 6 | 2 ± 5 | 3 ± 3 | 41 ± 10 | 22 ± 6 | 3 ± 6 | 3 ± 4 |
| Nordiazepam | 18 ± 11 | 9 ± 7 | 14 ± 11 | 12 ± 12 | 12 ± 10 | 4 ± 8 | 9 ± 8 |
| Nitrazepam | 3 ± 5 | 29 ± 24 | N.D. | 4 ± 3 | 1 ± 2 | 2 ± 2 | 4 ± 8 |
| Oxazepam | N.D. | N.D. | 184 ± 123 | 281 ± 198 | 73 ± 147 | 98 ± 195 | 83 ± 96 |
| 7-amino-nitrazepam | 2 ± 3 | 28 ± 28 | 1 ± 1 | 5 ± 8 | 5 ± 6 | 2 ± 3 | 13 ± 4 |
| Lorazepam | 59 ± 59 | N.D. | 28 ± 35 | 9 ± 18 | 33 ± 27 | N.D. | 5 ± 9 |
| Anhydroecgonine methyl ester | 5 ± 1 | 8 ± 2 | 8 ± 2 | 9 ± 2 | 8 ± 2 | 12 ± 1 | 12 ± 1 |
| E1-HMA | 43 ± 29 | N.D. | N.D. | N.D. | N.D. | 13 ± 26 | 45 ± 35 |
| E2-HMA | 46 ± 4 | 11 ± 22 | N.D. | N.D. | N.D. | 12 ± 24 | 32 ± 22 |
| E1-HMMA | 27 ± 7 | 14 ± 2 | 9 ± 1 | 7 ± 1 | 10 ± 2 | 23 ± 2 | 35 ± 3 |
| E2-HMMA | 21 ± 5 | 12 ± 3 | 10 ± 1 | 9 ± 1 | 10 ± 2 | 27 ± 4 | 33 ± 2 |
| DHMA | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Caffeine | 184819 ± 14657 | 135883 ± 11735 | 173852 ± 8241 | 171064 ± 8077 | 171958 ± 5199 | 169130 ± 5162 | 151231 ± 5249 |
| 1,7-Dimethylxanthine | 107717 ± 4786 | 85882 ± 2418 | 137196 ± 28326 | 114869 ± 46279 | 75413 ± 6759 | 107717 ± 12820 | 106272 ± 31432 |
| (-)-Nicotine | 6152 ± 4540 | 3340 ± 653 | 7810 ± 4460 | 8562 ± 7806 | 6375 ± 3844 | 4872 ± 244 | 5549 ± 1807 |
| Cotinine | 2137 ± 324 | 1882 ± 202 | 2116 ± 35 | 2071 ± 83 | 2194 ± 67 | 2266 ± 115 | 2437 ± 114 |
| Creatinine | 679 ± 51 | 326 ± 87 | 355 ± 53 | 379 ± 124 | 338 ± 79 | 250 ± 58 | 330 ± 59 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Codeine | 2475 ± 56 | 1914 ± 269 | 2235 ± 247 | 1984 ± 195 | 2079 ± 134 | 1964 ± 184 | 1929 ± 257 |
| Oxycodone | 11 ± 2 | 33 ± 30 | 16 ± 6 | 14 ± 8 | 15 ± 4 | 11 ± 3 | 18 ± 7 |
| Noroxycodone | 21 ± 18 | 33 ± 23 | 13 ± 16 | 15 ± 10 | 33 ± 5 | 35 ± 12 | 25 ± 6 |
| Hydrocodone | N.D. | 22 ± 26 | 14 ± 29 | 10 ± 20 | 11 ± 22 | 38 ± 45 | 10 ± 20 |
| Oxymorphone | 14 ± 12 | 46 ± 35 | 20 ± 4 | 18 ± 2 | 18 ± 12 | 12 ± 9 | 19 ± 3 |
| Dihydrocodeine | 449 ± 37 | 437 ± 83 | 442 ± 19 | 380 ± 40 | 427 ± 36 | 419 ± 61 | 406 ± 59 |
| Methadone | 54 ± 6 | 50 ± 11 | 54 ± 2 | 53 ± 2 | 56 ± 4 | 59 ± 4 | 51 ± 5 |
| EDDP | 126 ± 13 | 105 ± 10 | 117 ± 11 | 106 ± 6 | 123 ± 16 | 112 ± 18 | 122 ± 8 |
| E1-Venlafaxine | 94 ± 9 | 74 ± 12 | 86 ± 9 | 92 ± 12 | 94 ± 9 | 102 ± 3 | 105 ± 3 |
| E2-Venlafaxine | 122 ± 3 | 88 ± 8 | 102 ± 11 | 97 ± 11 | 101 ± 12 | 109 ± 3 | 92 ± 7 |
| Vardenafil | 7 ± 9 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E1-Norephedrine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E2-Norephedrine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E1-PMA | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E2-PMA | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| Normorphine | 148 ± 23 | 131 ± 79 | 154 ± 16 | 181 ± 69 | 193 ± 52 | 152 ± 30 | 145 ± 27 |
| Dihydromorphine | 23 ± 5 | 43 ± 4 | 25 ± 10 | 27 ± 3 | 32 ± 15 | 15 ± 5 | 24 ± 5 |
| D1-Tramadol | 704 ± 17 | 720 ± 30 | 740 ± 48 | 766 ± 12 | 692 ± 32 | 772 ± 40 | 798 ± 39 |
| D2-Tramadol | 640 ± 2 | 666 ± 21 | 678 ± 13 | 621 ± 65 | 672 ± 26 | 651 ± 37 | 595 ± 22 |
| *O*-Desmethyltramadol | 836 ± 76 | 873 ± 25 | 950 ± 21 | 882 ± 20 | 801 ± 16 | 849 ± 3 | 860 ± 9 |
| Zolpidem | 1 ± 1 | 1 ± 2 | N.D. | N.D. | N.D. | N.D. | N.D. |
| Amitriptyline | 234 ± 40 | 126 ± 17 | 257 ± 29 | 227 ± 13 | 232 ± 59 | 218 ± 6 | 245 ± 22 |
| Norketamine | 47 ± 10 | 39 ± 9 | 32 ± 4 | 57 ± 1 | 50 ± 14 | 45 ± 7 | 37 ± 8 |
| Sildenafil | 30 ± 9 | 3 ± 0 | 21 ± 7 | 13 ± 0 | 12 ± 2 | 11 ± 2 | 20 ± 7 |
| (+)-Ephedrine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| (-)-Ephedrine and (-)-Ψephedrine | 23 ± 10 | 21 ± 3 | 28 ± 4 | 24 ± 3 | 27 ± 3 | 18 ± 7 | 17 ± 2 |
| (+)-Ψephedrine | 201 ± 15 | 191 ± 10 | 169 ± 22 | 163 ± 20 | 160 ± 19 | 136 ± 13 | 153 ± 3 |
| Desmethylvenlafaxine-E1 | 291 ± 2 | 296 ± 13 | 292 ± 14 | 289 ± 10 | 315 ± 7 | 269 ± 15 | 305 ± 15 |
| Desmethylvenlafaxine-E2 | 250 ± 6 | 233 ± 9 | 235 ± 8 | 191 ± 4 | 225 ± 15 | 211 ± 12 | 215 ± 26 |
| E1-Zopiclone | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E2-Zopiclone | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| *S*-(+)-fluoxetine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| *R*-(-)-fluoxetine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E1-Norfluoxetine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| E2-Norfluoxetine | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |

N.D - not detected

**Table 3-9** In-sewer stability of illicit drugs and potentially abused licit drugs through chiral HPLC-MS/MS (QqQ) analysis (n.d. means <MDL).

| Analyte | Day-to-day variation in concentrations % (n=3) | | In-sewer stability Day 1 [%] ± SD | In-sewer stability Day 2 [%] ± SD | In-sewer stability Day 3 [%] ± SD | In-sewer stability (Average [%] ± SD) |
|---|---|---|---|---|---|---|
| | Inlet | Outlet | | | | |
| Cocaine | 29.5 | 33.3 | 31.4 | 11.0 | 21.8 | 21.4 ± 10.2 |
| Benzoylecgonine | 24.0 | 27.8 | 10.6 | 6.9 | 21.7 | 13.0 ± 7.7 |
| Cocaethylene | 20.6 | 16.1 | 20.1 | 7.6 | 34.6 | 20.8 ± 13.5 |
| *R*-(-)-Amphetamine | 13.6 | 31.6 | 4.9 | 7.9 | 38.0 | 13.7 ± 22.0 |
| *S*-(+)-Amphetamine | 10.7 | 22.6 | 2.2 | 5.5 | 44.3 | 13.7 ± 26.8 |
| *R*-(-)-Methamphetamine | 113.9 | 109.6 | 18.7 | 15.6 | 61.3 | 31.9 ± 25.5 |
| *S*-(+)-Methamphetamine | 22.8 | 33.5 | 21.8 | 17.8 | 4.7 | 11.6 ± 14.3 |
| E1-Mephedrone | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E2-Mephedrone | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *R*-(-)-MDA | 28.2 | 33.6 | 62.8 | 1.5 | 16.5 | 16.0 ± 41.6 |
| *S*-(+)-MDA | 31.5 | 34.1 | 14.8 | 10.7 | 21.4 | 15.6 ± 5.4 |
| *R*-(-)-MDMA | 52.6 | 44.1 | 7.2 | 4.0 | 14.4 | 3.7 ± 10.8 |
| *S*-(+)-MDMA | 57.9 | 38.5 | 13.8 | 7.1 | 43.5 | 7.5 ± 31.3 |
| E1-MDEA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E2-MDEA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *O*-6-monoacetylmorphine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Benzylpiperazine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Temazepam | 10.9 | 3.6 | 26.3 | 22.3 | 14.6 | 21.1 ± 6.0 |
| Diazepam | 13.2 | 19.2 | 24.2 | 0.0 | 29.6 | 1.8 ± 27.0 |
| Nordiazepam | 32.4 | 14.5 | 35.9 | 19.6 | 7.9 | 8.1 ± 27.8 |
| Nitrazepam | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Oxazepam | 4.2 | 19.1 | 6.0 | 0.3 | 32.6 | 12.8 ± 17.5 |
| 7-amino-nitrazepam | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Lorazepam | 1.9 | 7.8 | 14.9 | 10.4 | 26.3 | 17.2 ± 8.2 |
| E1-HMA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E2-HMA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

| | | | | | | |
|---|---|---|---|---|---|---|
| DHMA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Caffeine | 14.1 | 22.7 | 39.2 | 29.2 | 51.9 | 40.1 ± 11.4 |
| 1,7-dimethylxanthine | 4.9 | 9.6 | 46.4 | 34.8 | 50.6 | 44.0 ± 8.2 |
| Nicotine | 16.8 | 21.7 | 20.0 | 4.5 | 34.9 | 19.8 ± 15.2 |
| Cotinine | 3.1 | 2.5 | 0.1 | 0.5 | 1.3 | 0.6 ± 0.7 |
| Creatinine | 23.9 | 5.3 | 24.0 | 1.2 | 6.4 | 6.3 ± 15.8 |
| Codeine | 13.6 | 21.8 | 9.5 | 6.0 | 13.1 | 3.2 ± 11.5 |
| Oxycodone | 4.8 | 12 | 1.2 | 7.5 | 23.9 | 10.1 ± 12.7 |
| Noroxycodone | 2.7 | 26.7 | 7.4 | 24.4 | 44.4 | 25.4 ± 18.6 |
| Hydrocodone | 22.2 | 20.4 | 27.3 | 34.8 | 3.2 | 3.6 ± 31.0 |
| Oxymorphone | 15.9 | 18.9 | 13.3 | 13.0 | 37.0 | 12.4 ± 25.0 |
| Dihydrocodeine | 26.4 | 31.0 | 10.5 | 3.1 | 0.0 | 4.6 ± 5.4 |
| Methadone | 13.2 | 13.0 | 22.1 | 0.9 | 14.3 | 11.8 ± 11.7 |
| EDDP | 22.5 | 28.7 | 8.9 | 1.1 | 4.0 | 2.0 ± 6.5 |
| E1-Venlafaxine | 3.6 | 18.8 | 1.2 | 13.4 | 27.7 | 14.1 ± 13.3 |
| E2-Venlafaxine | 7.1 | 21.2 | 10.0 | 16.5 | 33.3 | 19.9 ± 12.1 |
| Vardenafil | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E1-Norephedrine | 20.9 | 26.4 | 76 | 9.8 | 18.8 | 22.4 ± 48.6 |
| E2-Norephedrine | 28.5 | 31.1 | 22.7 | 7.8 | 1.3 | 9.7 ± 12.1 |
| E1-PMA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E2-PMA | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Dihydromorphine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Zolpidem | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Amitriptyline | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Sildenafil | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E1-Zopiclone | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E2-Zopiclone | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *S*-(+)-Fluoxetine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *R*-(-)-Fluoxetine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E1-Norfluoxetine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| E2-Norfluoxetine | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

## 3.5 Conclusions

Understanding patterns of drug use is of key importance in public health monitoring. WBE, a new non-intrusive tool, provides significant advances in the field. It allows for multiple temporal and spatial drug use estimates in near-real time. Enantiomeric profiling provides a new dimension to WBE as it can help with the verification of the origin of drug residue, potency of abused drug and its synthetic route. To aid enantiomeric profiling in WBE, a new analytical method utilising a CBH column and liquid chromatography coupled with tandem mass spectrometry was developed. The method showed very good performance: >90% SPE recoveries, very good sensitivity (MDLs and MQLs at ppt levels), high linearity range and method precision <10%. The method allowed for the analysis of 56 drug biomarkers in wastewater. These are: opioid analgesics, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, PDE5 inhibitors, amphetamine and methamphetamine drug precursors. Satisfactory enantiomeric separation was obtained for 18 pairs of enantiomers including amphetamine, methamphetamine, MDMA and its metabolites HMA and HMMA, PMA, MDA and mephedrone. The method was applied in a one week monitoring study of a large wastewater treatment plant in the UK. Most target drugs were found at quantifiable concentrations in analysed samples. The results for several drugs such as cocaine and MDMA and their metabolites showed a clear trend of increased concentrations during weekend. Enantiomeric profiling revealed that amphetamine, methamphetamine and MDMA were found enriched with *R*-(-)-enantiomers, probably due to their stereoselective metabolism favouring *S*-(+)-enantiomers. MDA was either enriched with *R*-(-)- or *S*-(+)-enantiomer indicating that its presence might be due to either abuse of racemic MDA or abuse of racemic MDMA. Non-racemic enantiomeric fractions were also observed in the case of HMMA and mephedrone suggesting enantioselective metabolism. To the author's knowledge, this is the first time chiral separation and wastewater profiling of mephedrone, PMA, MDMA and its metabolites HMA and HMMA is reported. In-sewer stability study performed in a pressurized sewer under anaerobic conditions showed that nearly 44% of the compounds were highly stable with changes in concentrations within 20%.

## 3.6 Contributions

In-sewer stability study was carried out by Dr. Oriol Gutierrez and Olga Auguet.

## 3.7 Supplementary Data

The following supplementary data are contained in Appendix 1:

Table S1 Selected analytes and their properties.

Table S2 Studied mobile phase compositions with CHIRALPAK® CBH HPLC.

Table S3 Studied mobile phase compositions with CHIROBIOTIC V.

Table S4 Studied mobile phase compositions with CHIROBIOTIC T.

Table S5 Validation parameters -instrumental precision.

Table S6 Validation parameters- ion suppression.

Figure S1 CBH column - enantiomeric resolution of studied analytes in a mobile phase containing acetonitrile as organic modifier (mobile phase composition: 1mM ammonium acetate/acetonitrile 9:1).

Figure S2 CBH column - enantiomeric resolution of studied analytes in a mobile phase containing isopropanol as organic modifier (mobile phase composition: (a) 1mM ammonium acetate/isopropanol 9:1 and (b) 1mM ammonium acetate/isopropanol 9.5:0.5).

Figure S3 CBH column - enantiomeric resolution of studied analytes in mobile phases containing: (a) 1 mM ammonium acetate/methanol 9.5:0.5, (b) 1 mM ammonium acetate/methanol 9:1, (c) 2.5 mM ammonium acetate/methanol 9:1, (d) 5 mM ammonium acetate /methanol 9:1, (e) 10 mM ammonium acetate /methanol 9:1 and (f) 1 mM ammonium acetate /methanol 8.5:1.5.

Figure S4 CBH column - Impact of different percentanges of modifiers on retention time of analytes.

Figure S5 Chirobiotic T column - overview of the separation for the targeted analytes

Figure S6 Chirobiotic T column - separation of oxazepam and lorazepam.

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# Chapter 4: A new approach towards biomarker selection in estimation of human exposure to chiral drugs with limited metabolism data: a case study of mephedrone

## 4.1 Summary

WBE is an innovative approach that utilises biomarker analysis in wastewater with the aim of estimating public health status. A new compound detected in wastewater can be a potential biomarker and an indicator of a new emerging trend in public health. However, it is currently very difficult to select and validate new biomarkers for NPSs use mainly due to limited or unavailable human metabolism data. This chapter presents a new framework that enables the identification and selection of new biomarkers of human exposure to drugs with limited or unknown human metabolism data. Mephedrone was chosen as a target compound in this study to elucidate the assessment of biomarkers for a new

emerging drug of abuse using a multi-step analytical procedure. The developed framework consists of four steps: (i) the identification of possible metabolic biomarkers present in wastewater using in-direct *in-vivo* study; (ii) the verification of chiral signature of the target compound; (iii) the confirmation of human metabolic residues in *in-vivo* and *in-vitro* studies and (iv) the verification of stability of possible biomarkers in wastewater. Mephedrone was selected as a suitable biomarker due to high stability profile in wastewater. Its enantiomeric profiling was studied for the first time in several biological and environmental matrices, showing that chiral analysis was fundamental in order to distinguish human consumption from possible direct disposal of unused mephedrone. Further biomarker candidates for WBE approach were also proposed for future studies. These are: 4'-carboxy-mephedrone, 4'-carboxy-normephedrone, 1-dihydro-mephedrone, 1-dihydro-normephedrone and hydroxyl-tolyl-normephedrone.

## 4.2 Introduction

WBE is a new approach that uses biomarker analysis in wastewater with the aim of understanding, estimating and monitoring population health and lifestyle. WBE is being currently applied to monitor spatial and temporal illicit drug usage at local, national and international scale [1-12]. A wider list of biomarkers including cocaine, benzoylecgonine, amphetamine, methamphetamine, MDMA, 11-nor-9-carboxy-delta(9)-tetrahydrocannabinol and other compounds (e.g. heroin, 6-MAM, morphine, mephedrone, ketamine, GHB) has been recently proposed in order to achieve a more comprehensive estimation of drug abuse at community level [13].
A new compound that was detected for the first time in wastewater via non-target high resolution mass spectrometry (HRMS) screening can be considered as a potential biomarker and an indicator of a new emerging trend in public health and lifestyle. This is of particular importance in the identification and monitoring of the emergence of new psychoactive substances (NPSs). Unfortunately, it is very difficult to validate new biomarkers mainly due to limited or unavailable human metabolism data. This chapter presents a new framework that enables the identification and selection of new biomarkers of human exposure to drugs with limited or unknown human metabolism data. Mephedrone was chosen in this study to elucidate the assessment of biomarkers for a new emerging drug of abuse using a multi-step analytical procedure.

Mephedrone is a stimulant semisynthetic derivative of cathinone. It was first synthesised in 1929 by Saem de Burnaga Sanchez but its abuse has been documented for the first time only in 2007 [14]. Abuse of mephedrone was reported in several European countries. Recently, several mephedrone abuse associated deaths were reported in the UK [15]. In response to this, several modified cathinones were included in the UK Misuse Drugs Act (in category class B) in April 2010. Four fatalities due to mephedrone intake were confirmed in Scotland between February and May 2010 [16].

Mephedrone is a chiral compound. It contains one chiral carbon and it exists in two enantiomeric forms as *R*-(+)-mephedrone and *S*-(-)-mephedrone. Mephedrone can be synthesised via both non-stereoselective and stereoselective methods as shown in Figure S1, but, as reported by EMCDDA [17], 'street mephedrone' is most probably distributed as racemate. Routes of administration include oral administration, snorting, rectal or intravenous administration. Metabolism of mephedrone in humans and rats was investigated by several research groups [18] [14] [19] [20]. The metabolism in humans was verified by Pozo et al. 2015 [21] using an *in vivo* study in two volunteers. Six phase I and four phase II metabolites were reported in urine (Figure S2). Normephedrone and 4-hydroxytolylmephedrone, which are two phase I metabolites, showed biological activity serving as substrates at monoamine transporters [22]. Stereoselectivity of mephedrone was hardly investigated. Stereospecific effects of mephedrone enantiomers in rats were reported by Gregg et al. [23]. *R*-(+)-mephedrone showed predominant dopaminergic action and more stimulant-like properties than *S*-(-)-mephedrone [24].

Mephedrone was reported by EMCDDA (EU Early Warning System) to have increased usage in the UK in 2014 [25]. Its purity showed a decreasing trend in South Wales since its ban in the UK (68.2%± 24.9% as mean value ± SD) [26]. It was also detected and quantified in wastewater in Cambridge (UK) [27] and during a week monitoring campaign in the UK in 2014 [28]. There is very limited information regarding mephedrone in wastewater. It was found in wastewater of ten Chinese megacities at levels < 2.8 mg/1000 inhabitants $day^{-1}$ [19] and in only two Italian cities over a four-year monitoring study, which confirms its low use in Italy [29]. In all studies, the drug target residue (DTR) for WBE estimations was the parent compound mephedrone due to very limited information on human

metabolism. This constitutes an issue for the WBE approach, as lack of metabolic DTRs does not allow for accurate verification of drug use (e.g. distinction between drug consumption and disposal of unused drug). To solve this problem, this chapter proposes a novel comprehensive framework that enables biomarker selection in WBE for new drugs of abuse with limited knowledge of human metabolism.

## 4.3 Experimental

### 4.3.1 Chemical and Materials

Table S1 shows target analytes, their CAS number, molecular formula, molecular weight, log P, purity and supplier information. The deuterated analogue mephedrone-$D_3$ was used as internal standard (IS). All standards and IS were of the highest purity available (≥98%). Stock and working solutions of standards were stored at -20° C. Methanol, acetonitrile and ammonium acetate were purchased from Sigma Aldrich, UK. Ultrapure water was obtained from PURELAB UHQ-PS Unit (Elga, UK). The deactivation of the glassware was carried out in order to prevent the adsorption of mephedrone and its metabolites to the hydroxyl sites of the glass surface. The process consisted of the following steps: rinsing of the glassware with 5% DMDCS once, toluene twice and methanol thrice. Glucuronic acid (CAS 6556-12-3, Sigma Aldrich, UK) and active sulphate adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate, known as PAPS (CAS 109434-21-1, Sigma Aldrich, UK), were used as substrates for the investigation of the phase II metabolism of mephedrone.

### 4.3.2 Sample collection, storage and sample preparation

#### 4.3.2.1 Street mephedrone samples

Eight street mephedrone powder samples were collected from amnesty bins at one of the festivals in the UK in 2014. Methanolic solutions were prepared and stored in a freezer at -20°C. Diluted solutions in 1 mM ammonium acetate/methanol 85:15 v/v were spiked with a solution of mephedrone-$D_3$ at 1 μg $mL^{-1}$ and injected in the chiral liquid chromatograph coupled with triple quadrupole system (chiral LC TQD).

#### 4.3.2.2 Rat urine samples

Metabolism of mephedrone in rats was investigated at Saarland University. 20 mg $kg^{-1}$ body mass dose of (±)-mephedrone was administered orally to a male Wistar rat (Charles River, Sulzfeld, Germany) for toxicological diagnostic reasons according to the corresponding German law (http://www.gesetze-im-internet.de/tierschg/). The rat was kept in a metabolism cage for a day having water *ad libitum*. Rat faeces and urine samples were separated during the 24 hours of collection time and stored at -20 °C in a freezer. Blank rat urine samples collected before drug administration were used as control samples. Collected urine samples were diluted 100-fold and directly injected into high performance liquid chromatography coupled to high resolution mass spectrometry (HPLC-HRMS) system: an Orbitrap Q-Exactive (LC Q-E). Acetylation of rat urine sample was carried out in order to verify the presence of an acetyl group in a mephedrone metabolite. Experimental settings and procedure are described in the appendix 2 (S1). In order to undertake chiral LC TQD analysis, samples were reconstituted in 100 µL of 1 mM ammonium acetate/methanol 85:15 v/v. Two standard addition curves for the quantification of mephedrone and normephedrone in rat urine samples were prepared at seven concentration levels.

#### 4.3.2.3 Pooled urine samples

Seven pooled urine samples were collected in August 2014 from a UK festival event. They came from five different urinals sampled on three different days. 3 mL of each sample were spiked with 50 µL of mephedrone-$D_3$ at 1 µg $mL^{-1}$ and underwent SPE using Oasis HLB cartridges (60 mg, Waters, UK) as described in paragraph 4.3.2.4 (for "Monitoring campaigns"). Liquid-liquid extraction (LLE) was then performed using ethyl acetate and sodium phosphate at pH 8-9. Samples were centrifuged for 5 minutes at 5000 rpm. The supernatant was evaporated to dryness under nitrogen flow at 40°C and reconstituted in 250 µL of 1mM ammonium acetate/methanol 85:15 v/v. After being filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm), 20 µL were injected into the chiral LC TQD system.

#### 4.3.2.4 Wastewater samples

**Monitoring campaigns**

24h time-proportional (10 mL every 15 minutes) composite wastewater influent samples were collected in PTFE bottles from a local wastewater treatment plant. They were then transported to the laboratory in cool boxes packed with ice blocks and filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK). 100 µL of a mixture of IS at concentration 1 mg $L^{-1}$ were added to 100 mL of a wastewater sample to give final concentration of 1 µg $L^{-1}$.

SPE was carried out using Oasis HLB cartridges (60 mg, Waters, UK). The cartridges were conditioned with 2 mL of methanol followed by equilibration with 2 mL of ultrapure water at a rate of 3 mL $min^{-1}$. 100 mL of wastewater (spiked with IS at 1 µg $L^{-1}$) were passed through the HLB cartridge at a rate of 8 mL $min^{-1}$. The cartridges were then washed with 3 mL of ultrapure water at a rate of 3 mL $min^{-1}$ and the analytes were eluted with 4 mL of methanol at a rate of 8 mL $min^{-1}$ into 5mL silanised glass tubes. The extract was transferred to the TurboVap evaporator (Caliper, UK). After evaporation to dryness under nitrogen flow (5-10 psi) at 40 °C the samples were reconstituted in 0.5 mL 1 mM ammonium acetate/methanol 85:15 v/v and filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm). The filtered samples were transferred to polypropylene plastic vials bonded pre-slit PTFE/Silicone septa (Waters, UK) and then 20 µL were directly injected into the chiral LC TQD system.

**Stability of mephedrone in wastewater**

Stability of mephedrone and normephedrone in wastewater was investigated in dark biotic reactors (containing wastewater spiked with the analyte) at 4°C and 17°C for a duration of 48 hours. 500 mL of wastewater were spiked in duplicate with 1 µg $L^{-1}$ of mephedrone or normephedrone. Unspiked wastewater reactors were also included as controls. 50 mL of wastewater samples were collected at 0, 12, 24 and 48 hours, and spiked with IS. pH and temperature were constantly monitored. Samples were filtered through GF/F glass fiber filters and solid-phase extracted as described in the section above (paragraph 4.3.2.4-"Monitoring campaigns"). Samples were eluted with 4 mL of methanol, dried under nitrogen flow at 40 °C and reconstituted in 0.5 mL of 1 mM ammonium acetate/methanol 85:15 v/v for the chiral LC TQD analysis and in 0.5 mL of methanol for their LC Q-E analysis.

### Incubation of mephedrone in wastewater and formation of metabolites

Mephedrone was incubated in the following reactors: biotic (containing wastewater spiked with the analyte), abiotic (containing the wastewater spiked with the analyte and sodium azide for quenching any bacterial growth), clean (containing demineralised water spiked with the analyte and sodium azide) and control (only wastewater) (Table S2a). 4 mL $NaN_3$ solution (50 g $L^{-1}$) were added to clean and abiotic reactors (containing 400 mL of wastewater each) yielding a concentration of 0.2% v/v $NaN_3$. Sampling was performed at time 0, 4 and 7 days. The sample preparation was performed using the procedure with the QuEChERs devices published elsewhere [30]. Briefly, 1.6 g $MgSO_4$, 0.4 g NaCl and 4 mL IS (in this case 250 μL of 10 μg $mL^{-1}$ mephedrone-$D_3$ were diluted in 50 mL of acetonitrile) were mixed. 4 mL from incubated reactors were added and shaken vigorously. After centrifugation at 5 °C for 5 minutes at 1200 rpm, 1.5 mL of supernatant were transferred to the QuEChERs kit. Samples were centrifuged again for 5 minutes at 14680 rpm. 1 mL was evaporated to dryness under nitrogen flow at 40 °C. Samples were then injected in the LC Q-E system. In order to detect and identify biotransformation products of the incubated wastewater, SPE was performed using Biotage HCX cartridges, previously conditioned with 1mL of methanol followed by 1mL of deionised water. 3 mL of filtered and spiked with mephedrone-$D_3$ wastewater from each reactor were loaded into the cartridges. 1mL of deionised water followed by 1mL of 0.01 M HCl and 1mL deionised water were used for washing the cartridges. 2 mL of methanol were used for eluting the neutral fraction, whilst 1mL methanol/$NH_3$ 33% mix 98:2 v/v for the basic fraction. Analysis of data dependent MS/MS fragmentation (ddMS2) was performed. The software used were EAWAG-BBD Pathway Prediction System (http://eawag-bbd.ethz.ch/predict/) and XCMS Online by the Scripps Research Institute (https://xcmsonline.scripps.edu/).

### 4.3.2.5 Pooled human liver microsomes (pHLM) experiments

Two experiments were performed for the *in vitro* metabolism studies of mephedrone. The first one (A) was focused only on metabolites from phase I metabolism, whilst the second (B) also took into account phase II conjugated metabolites.

*Experiment A.* (±)-Mephedrone was incubated at a final concentration of 2.5 μM over 60 minutes in biological triplicate reactors. The reactors are described in Table S2b. 100 μL of each reactor contained 90 mM of phosphate buffer at pH 7.4, 25 μM of substrate solution, 20 U $mL^{-1}$ of Superoxide Dismutase (SOD). Apart from "No HLM" reactor, 1 mg $mL^{-1}$ of pooled human liver microsomes (pHLM) (BD Biosciences, Heidelberg, Germany) was added to all reactors. The regenerating system consisted of isocitrate, $MgCl_2$ and $NADP^+$ (Biomol, Hamburg, Germany). Sampling points were set at 0, 10, 20, 30 and 60 minutes. Time 0 was set when ice cold pHLM was added in the reactors for starting the reaction, whilst at time 60 minutes ice cold acetonitrile was added to stop the reaction. Mephedrone-$D_3$ was also added in ice cold acetonitrile to all the incubated samples at a concentration of 100 ng $mL^{-1}$. Samples were shaken thoroughly and left in the freezer for 5 minutes. pHLM samples were liquid-liquid extracted (LLE) with 300 μL ethyl acetate (pH 8-9 adding sodium phosphate). Samples were then centrifuged for 5 minutes at 14680 rpm. The supernatant was gently evaporated to dryness under nitrogen flow. Finally, samples were reconstituted in 55 μL of 1mM ammonium acetate/methanol 85:15. Five microliters were injected in chiral LC VP system.

*Experiment B.* Mephedrone was incubated at a final concentration of 10 μM over 180 minutes in biological duplicates. A single reactor was made of: analyte solution, a buffer solution, containing 50 mM $KH_2PO_4$ and 5 mM $MgCl_2$ at pH 7.4, a NADPH 50 mM solution and pHLM. Glucuronic acid and PAPS were used as substrates for the investigation of Phase II metabolism reactions, such as glucuronidation and sulfation respectively. Sampling points were set at 0, 10, 20, 30, 60 and 180 minutes for mephedrone incubation and at 180 minutes for phase II metabolism investigation. In this experiment, the blank contained the analyte but not the pHLM. Mephedrone-$D_3$ was also added in ice cold acetonitrile to all the incubated samples for stopping the reaction. Samples were shaken thoroughly and centrifuged for 10 minutes at 12000 rpm. Samples for the investigation of the phase II metabolism were evaporated at 40 °C and reconstituted in 100 μL of water/methanol 8:2. Ten microliters were injected in liquid chromatography coupled with quadrupole time-of-flight (LC QTOF) system. LLE of the pHLM samples incubating normephedrone was performed with 300 μL ethyl acetate (pH 8-9 adding sodium phosphate). Samples were then centrifuged for 5 minutes at

14680 rpm. The supernatant was evaporated to dryness under nitrogen flow. Samples were reconstituted in 500 µL of 1mM ammonium acetate/methanol 85:15 v/v. 20 µL were injected in chiral LC TQD system.

### 4.3.3 Sample analysis with liquid chromatography coupled with tandem mass spectrometry

#### 4.3.3.1 Quantification of mephedrone and its metabolites using targeted quantitative analysis utilising chiral liquid chromatography coupled with triple quadrupole (chiral LC TQD)

Separation of all the analytes was undertaken with the validated methodology using chiral LC TQD according to [28]. Briefly, a Waters ACQUITY UPLC® system (Waters, Manchester, UK), a cellobiohydrolase column (CBH) CHIRALPAK® CBH HPLC Column 5 µm particle size, L × I.D. 10 cm × 2.0 mm (Chiral Technologies, France) with a Chiral-CBH guard column 10 × 2.0 mm, 5 µm particle size (Chiral Technologies, France) coupled with a triple quadrupole mass spectrometer Xevo TQD (Waters, Manchester, UK) equipped with an electrospray ionisation source (ESI) were used. ACQUITY UPLC™ autosampler was kept at 4 °C, while the column temperature was set at 25 °C. The injection volume of the sample was set at 20 µL. The mobile phase was 1mM ammonium acetate/methanol 85:15 v/v at a 0.1 mL $min^{-1}$ under isocratic conditions. The system operated in positive mode with a capillary voltage of 3 kV, source temperature at 150 °C, desolvation temperature at 265 °C and desolvation gas flow at 550 l $h^{-1}$. Nitrogen, supplied by a high purity nitrogen generator (Peak Scientific, UK), was used as a nebulising and desolvation gas. Argon (99.999%) was used as a collision gas. MassLynx (Waters, UK) was used to control the Waters ACQUITY system and the Xevo TQD. Data processing was carried out on TargetLynx 4.1 software (Waters, Manchester, UK). MRM transitions of the studied compounds, cone voltages and collision energies are summarised in Table S3. In Table S4 are shown validation parameters, such as instrumental and method limits of detection and quantification, linearity (Table S4a), SPE recovery (Table S4b), method precision (Table S4c), instrumental precision (Table S4d), resolution of enantiomers and enantiomeric fraction (Table S4e). The mean correlation coefficients ($R^2$) of the calibration curves were ≥ 0.9990 for the investigated compounds. The analytes showed linearity from 0.25 µg $L^{-1}$ up to 500 µg $L^{-1}$ for single enantiomer highlighting the

high level of performance of the CBH column for these compounds. The instrumental and method limits of detection were respectively 0.25 µg $L^{-1}$ for both analytes and nearby 1 ng $L^{-1}$, whilst instrumental and method limits of quantification were in both cases lower for mephedrone than for normephedrone. The identification criteria for each analyte were in accordance to European guidelines [31].

#### 4.3.3.2 Identification of metabolites using targeted and non-targeted analysis with high performance liquid chromatography coupled with high resolution mass spectrometry (HPLC-HRMS)

The analyses were performed using three different HRMS systems: LC VP, LC Q-E and LC QTOF respectively.

*Orbitrap system Velos Pro (LC-VP).* The separation of the analytes was undertaken with UltiMate 3000 HPLC system (Thermo Scientific, Germany) and a CHIRALPAK® CBH HPLC Column 5 µm particle size, L × I.D. 10 cm × 2.0 mm (Chiral Technologies, France) with a Chiral-CBH guard column 10 × 2.0 mm, 5 µm particle size (Chiral Technologies, France). UltiMate 3000 HPLC autosampler was kept at 4 °C, while the column compartment at 25 °C. The injection volume of the sample was 20 µL. The chosen mobile phase used for the method was 1 mM ammonium acetate/methanol 85:15 v/v at a 0.1 mL $min^{-1}$ under isocratic conditions. The optimisation of the sensitivity for mephedrone and normephedrone standard solutions was carried out at different source positions, heater temperatures and values of sheath and auxiliary gases. All analytes were detected using a mass spectrometer (Orbitrap system Velos Pro, Thermo Scientific, Germany) equipped with a heated electrospray ionisation source (H-ESI) operating in positive mode. It operated measuring the transition of the isolated protonated molecular ions of each compound in full scan mode and scanning the product ions spectra at m/z required at unit resolution in the full-scan MS/MS mode. ChromeLeon software was used to control both systems. Data processing was carried out on Xcalibur 2.1 software (ThermoFisher Scientific Inc., San Jose, CA, USA).

*Orbitrap Q-Exactive (LC-Q-E).* The LC–HRMS system was composed of a ThermoFisher Scientific (Dreieich, Germany) Accela LC system consisting of a degasser, a quaternary pump and an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), an Accucore™ Phenyl-Hexyl HPLC Column 2.6 µm

particle size, L × I.D. 10 cm × 2.1 mm (Thermo Scientific, Germany) coupled to a ThermoFisher Scientific Q-Exactive system (ThermoFisher Scientific, Dreieich, Germany). Mobile phase A was a solution of 2 mM aqueous ammonium formate plus 0.1% formic acid at pH 3, whilst mobile phase B was a solution of 2 mM aqueous ammonium formate with acetonitrile:methanol (50:50, v/v; 1% water) plus 0.1% formic acid. The sample injection volume was 10 µL. The flow rate was set at 0.5 mL $min^{-1}$ for 10 min and at 0.8 mL $min^{-1}$ from 10 to 13.5 min. Mobile phase gradient was as follows: 0–1.0 min 99% A, 1–10 min to 1% A, 10–11.5 min hold 1% A, 11.5–13.5 min hold 99% A. The ThermoFisher Scientific Q-Exactive system equipped with a heated electrospray ionization (HESI)-II source operated in positive and negative ion modes. Source spray voltage was at 3.00 kV (positive polarity) and at −4.00 kV (negative polarity); heater temperature and ion transfer capillary temperature were set both at 320 °C; S-lens RF level was at 60.0; sheath and auxiliary gases were 60 and 10 arbitrary units, respectively. Data were acquired in full scan mode and a subsequent data dependent acquisition (DDA) mode over a mass range of 130–1000 m/z with a resolving power of 35000 FWHM, microscans of 1, automatic gain control (AGC) target at 1e6, maximum injection time (IT) of 120 ms. Data processing was carried out on ThermoFisher Xcalibur Qual Browser software version 3.0.63 software.

*QTOF system (LC-QTOF).* MaXis High-Definition (HD) Q-TOF system (Bruker, Coventry, UK) was equipped with an ESI source operating in positive and negative mode. It was coupled to a UltiMate 3000 HPLC system (Thermo Scientific, UK) and an ACQUITY UPLC BEH C18 Column 5 cm 2.1 mm × 1.7 µm particle size (Waters, UK). UltiMate 3000 HPLC autosampler was kept at 4°C, while the column compartment at 40 °C. The injection volume of the sample was 10 µL. The chosen mobile phases delivered at 0.4 mL $min^{-1}$ were: A – 1mM $NH_4F$ in MilliQ-water; B – MeOH. The gradient was set as follows: 0-3 min 5% B, 3-4 min 5-60% B, 4-14 min 60% B, 14-14.1 min 60-98% B, 14.1-17 min 98% B, 17-17.1 min 98-5% B, 17.1-20 min 5% B. The mass spectrometer measured the m/z transition of the isolated protonated molecular ions in full scan mode, whilst it provided all MS and MS/MS information independently of the precursor in broadband CID (bb-CID) acquisition switching between high and low collision energies. For source settings, capillary was set at 4500 V, the nebulizer gas at 3.0 bar, the dry gas at 11.0 L $min^{-1}$ and the dry temperature at 220°C. HRMS data processing was carried out on

ACD/Labs Metabolite Identification software (Met-ID). It allowed the research of metabolites generated by the regioselectivity algorithms of ACD/Percepta, matching with those present in the experimental dataset and identifying them through IntelliTarget Algorithm.

### 4.3.4 Absolute configuration determination of mephedrone using circular dichroism (CD) and computational study

Absolute configuration determination of mephedrone was undertaken using a Perkin Elmer Series 200 HPLC system (equipped with a temperature controlled autosampler and column compartment, pump and a UV/VIS detector) coupled with a Chirascan Circular Dichroism Spectrometer (Applied Photophysics) equipped with a quartz spectrophotometer cell type 585.3/Q/10 cuvettes with a path length of 10 mm for micro flow (Starna scientific). The operating conditions are given in Table S5. Separation of mephedrone enantiomers was achieved using a CBH column at 0.1 mL $min^{-1}$ under isocratic conditions using 1 mM ammonium acetate/methanol 85:15 v/v as a mobile phase. The background, represented by the mobile phase was subtracted from CD spectra. UV absorbance and CD spectra were acquired simultaneously at $\lambda_{max}$= 265 nm [30] (Figure S3). The predicted UV spectrum obtained from the computational study was slightly shifted due to solvation effects on the electronic transitions when compared with the UV mephedrone spectrum reported by Maskell et al. (2011) [32]. Computational study gave predicted CD spectra for (+)- and (-)-mephedrone (Figure S4a) and for (+)- and (-)-normephedrone (Figure S4b). In correspondence of the first maximum absorbance peak at 265 nm the first eluting mephedrone enantiomer rotated the plane of polarized light with a negative Cotton effect, whilst the second peak with a positive effect. Combining the information obtained from the experimental spectrum and the modelling study, it was possible to establish that *R*-(+)-mephedrone eluted as the first enantiomer, while *S*-(-)-mephedrone as the second. Due to similar behaviour of the metabolite, also *R*-(+)-normephedrone and *S*-(-)-normephedrone were assessed as the first and second eluting enantiomers under the chromatographic conditions used.

### 4.3.5 Statistical analysis

Statistical analysis of the obtained ER values was performed with Data Analysis from Excel 2013. Statistical evaluation was done with an unpaired t-test

by comparing EFs from wastewater analysis and EFs from the analytical standards injected during the validation. T-test and p-value were considered for all wastewater samples in both sampling campaigns and excluding outliers, such as Wednesday in 2014 and Monday in 2015, in which EF was equal and below 0.5. F-test was also performed for assessing if variances were equal/unequal for the application of the t-test used. A significance level of $p < 0.05$ was initially set, then levels up to $p < 0.001$ were also tested. Paired t-test was used for comparing two datasets of wastewater samples, whilst an unpaired t-test for verifying any significant difference between EFs from illegal mephedrone samples and EFs from the analytical standards in the validation. All t-tests and p-values can be found in appendix 2 (S2).

## 4.4 Results and Discussions

Two sampling campaigns undertaken in the UK in years 2014 and 2015 confirmed the presence of mephedrone in wastewater. Its concentrations varied from 32 to 114 ng $L^{-1}$ in 2014 and from 65 to 192 ng $L^{-1}$ in 2015 (Figure 4-1 and Table S6).

Population-normalised mass loads were calculated as described elsewhere [6]. Briefly, daily mephedrone loads (g $day^{-1}$) were obtained by multiplying measured concentrations (ng $L^{-1}$) in daily samples with the corresponding wastewater volumes (L $day^{-1}$). Mephedrone loads were then normalized by the population size of the catchment (mg 1000 $people^{-1}$ $day^{-1}$). Loads ranged from 7.6 to 26.3 mg 1000 $people^{-1}$ $day^{-1}$, with a mean value of 14.7 ± 7.2 mg 1000 $people^{-1}$ $day^{-1}$ in 2014, whilst they ranged from 14.9 to 47.7 mg 1000 $people^{-1}$ $day^{-1}$ with a mean value of 25.6 ± 12.0 mg 1000 $people^{-1}$ $day^{-1}$ in 2015 (Table S6). The trend observed for the population-normalised mass loads throughout a week showed the highest loads during the weekend, which suggests its recreational use. As stated by EMCDDA [17], mephedrone is probably consumed in multiple doses of 0.5-2 g per session by users rather than single dose of 5-250 mg due to short-lived effects. Even if a mean dose value of 1.25 g was considered, daily doses could not be back-calculated due to missing DTR excretion data. Furthermore, enantioselective analysis revealed that mephedrone present in wastewater was enriched with *R*-(+)-mephedrone, except for the racemate found on Saturday in 2015 and on Wednesday in 2014 and 2015.

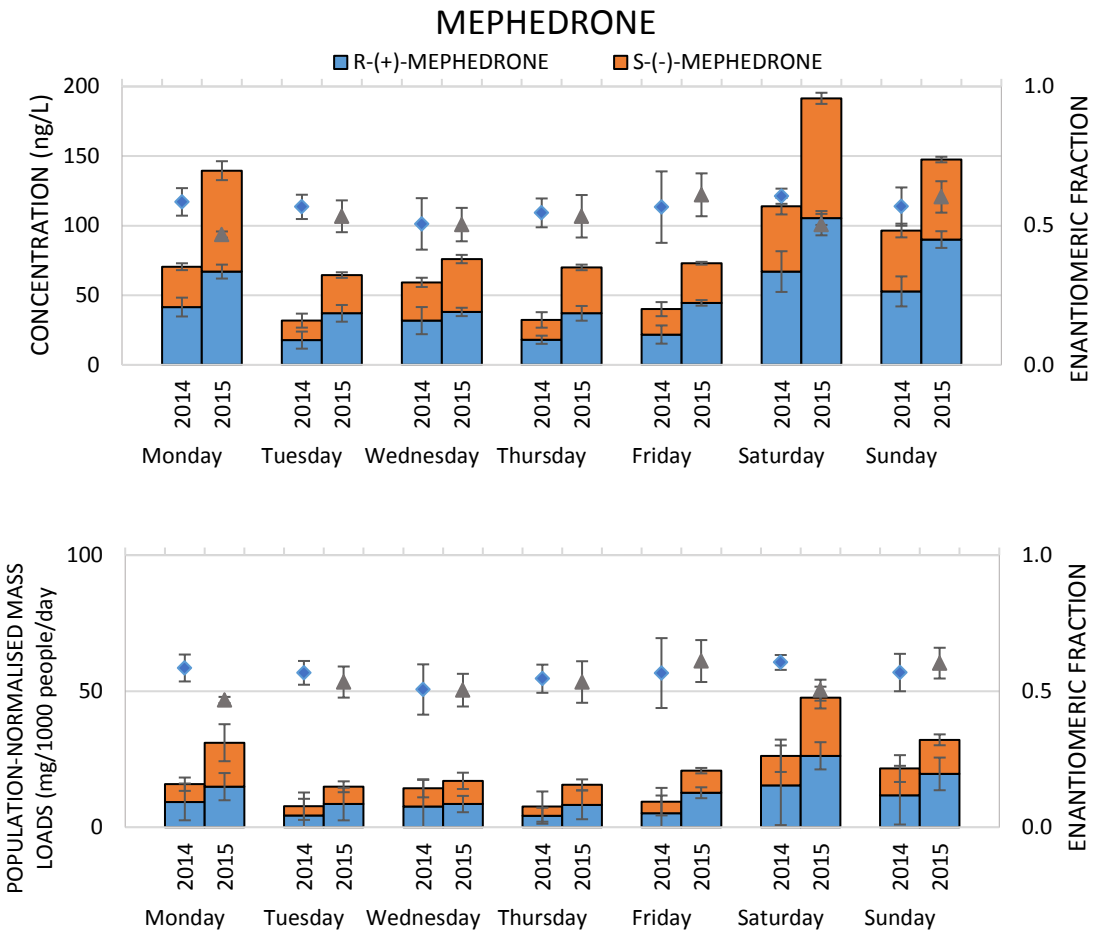



**Figure 4-1** Mephedrone in a week monitoring program. Results are displayed as concentrations (columns), population normalised-loads (columns) and enantiomeric fractions (symbols). Results obtained by applying the unpaired t-test showed "t Stat > t Critical one-tail" for all wastewater samples excluding Wednesday in 2014 (8.80 > 4.78), p one-tail (0.000024) < 0.001 and for all wastewater samples excluding Monday in 2015 (2.83 > 2.01), p one-tail (0.018) < 0.05. Therefore, EFs from wastewater samples were significant different (EF>0.5) from EF=0.5 during validation. Paired t-test results showed "t Stat < t Critical one-tail" (1.14 < 1.94), p one-tail (0.15) > 0.05, so two datasets of wastewater samples were not significant different from each other. All t-tests and P-values can be found in Appendix 2 (S2).

As reported by EMCDDA [17], mephedrone is distributed in Europe as racemate. Therefore, the presence of racemate in wastewater may indicate direct disposal. Enrichment of mephedrone with *R*-(+)-enantiomer can indicate stereoselective metabolism in humans and/or stereoselective microbial metabolic processes occurring in wastewater. However, due to lack of data on metabolism of mephedrone in humans and its fate in wastewater, no definite conclusions could be drawn regarding mephedrone abuse in the studied population.

Therefore, in this chapter, a robust analytical framework to enable accurate drug abuse estimation using WBE was proposed (Figure 4-2). The framework consists of four steps:

Step 1: Identification of possible metabolic biomarkers of mephedrone present in wastewater using LC-HRMS (in-direct *in-vivo* study).

Step 2: Verification of chiral signature of mephedrone using chiral liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

Step 3: Confirmation of metabolic residues in *in-vivo* (human and rat) and *in-vitro* (pHLM) studies.

Step 4: Microbial degradation in wastewater and verification of stability of possible mephedrone biomarkers in wastewater.

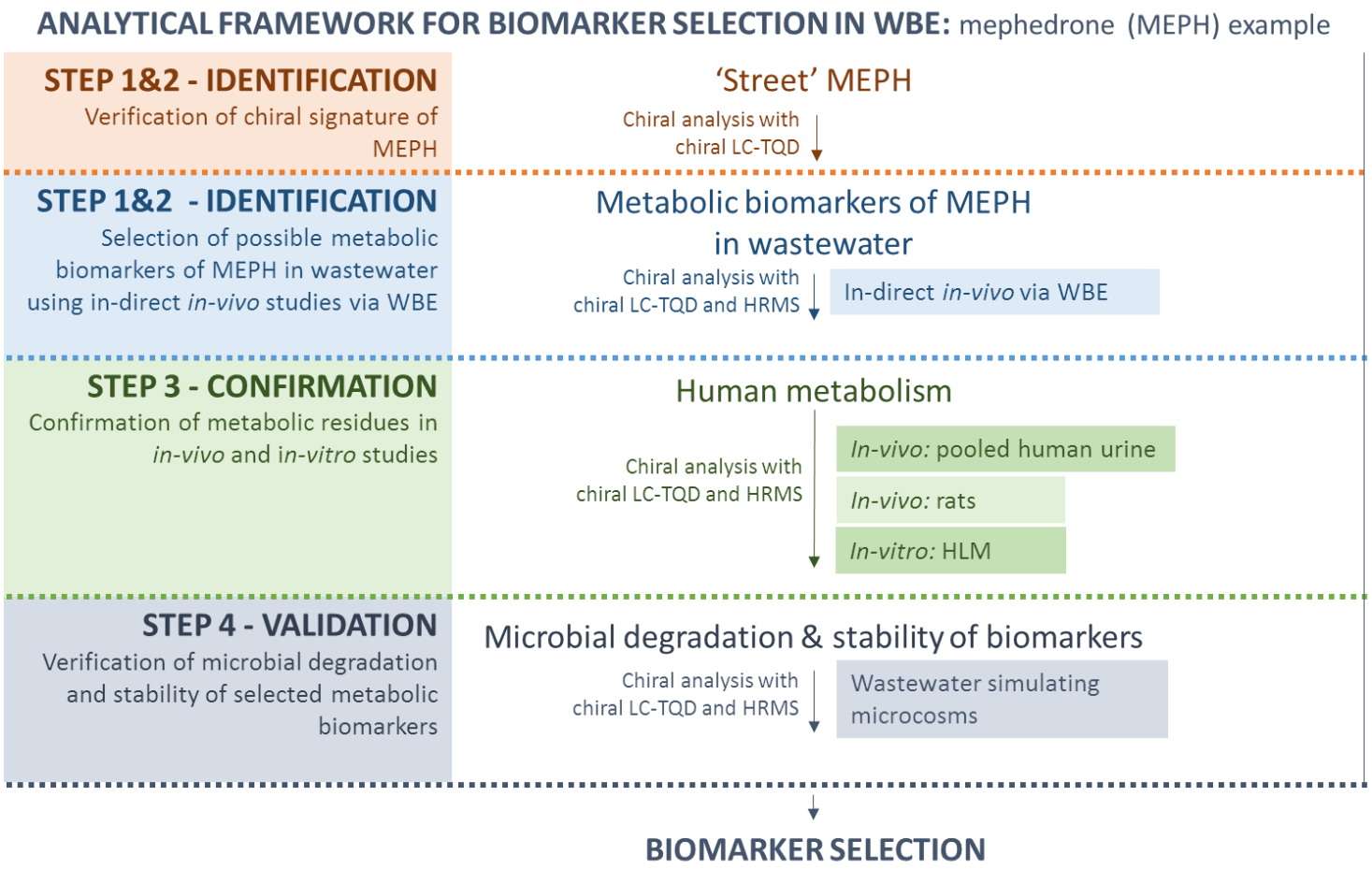



**Figure 4-2** Analytical framework proposed for the identification of suitable biomarkers of new occurring compounds for WBE approach (MEPH = mephedrone).

### 4.4.1 Step 1: Identification of possible metabolic biomarkers of mephedrone present in wastewater using LC-HRMS

Non-targeted analysis using MetID software enabled the prediction and the detection of the following metabolites: dihydro-mephedrone and *N*-sulfo-normephedrone, hydroxy-tolyl-normephedrone, 4'-carboxy mephedrone and 4'-carboxy normephedrone (Table 4-1 and S7, Figure 4-3). Target screening analysis in wastewater by using LC-QTOF did not confirm the presence of normephedrone as shown in Table S8. Non-targeted screening analysis allowed the detection of 1-dihydro-normephedrone with good mass accuracy for the precursor and the daughter ion with mass error <5ppm, one further daughter ion with an error <10ppm (Table S9). The precursor ion of the 4'-hydroxymethyl-mephedrone was detected at 6.2 min with -3.1 mass error. Through pHLM experiment, it was detected at 2.4 min with good mass accuracies for both the parent compound and the daughter ions. Even if the analytical standard of 4'-hydroxymethyl-mephedrone was not available for the final confirmation of the retention time, it was possible to state that 4'-hydroxymethyl-mephedrone was likely confirmed in the pHLM samples rather than in wastewater. 4'-carboxy-mephedrone was also found in wastewater, albeit it was not detected in the pHLM. Further work is, however, needed to verify whether any of the above biomarkers is a suitable DTR for estimation of mephedrone abuse via WBE.

### 4.4.2 Step 2: Verification of chiral signature of mephedrone with LC-TQD

Chiral signature of chemicals has already been proven invaluable in WBE in confirming consumption of MDMA [33], atenolol [34] and fluoxetine [35] vs their direct disposal. Chiral signature could also prove invaluable in the verification of potency of 'street drugs' as well as their synthetic routes. However, in order to apply such approach, the following two aspects need to be verified: (i) enantiomeric signature of distributed drug and (ii) possible changes in its enantiomeric signature during human metabolism.

#### 4.4.2.1 Step 2(a): 'Street' mephedrone

Chiral LC TQD analysis of the eight illegal mephedrone samples resulted in EF averaging at 0.50 ± 0.01 (Figure 4-4), which indicates non-stereoselective method

of synthesis. This confirms the conclusions of Gibbons and Zloh [36] and the EMCDDA report on mephedrone [17].

#### 4.4.2.2 Step 2(b): Metabolism of mephedrone in humans

As can be seen in Figure 1, mephedrone quantified in wastewater (with chiral LC TQD) was enriched with *R*-(+)-mephedrone. Knowing that mephedrone is distributed as racemate, it suggests that the presence of mephedrone in wastewater must have been subject to metabolic processes either in humans or other species such as microbes.

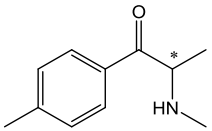
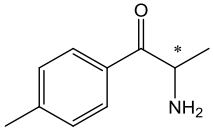
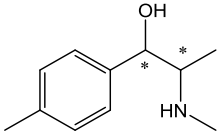
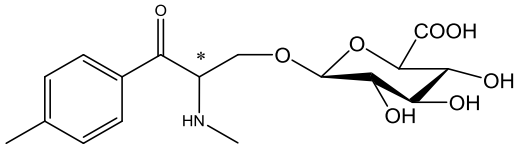
Unfortunately, as there is very limited knowledge of stereoisomerism of mephedrone, no conclusions can be drawn without any further studies. I therefore applied a multi-step approach in order to verify (a) the stereoselective metabolism of mephedrone in humans and (b) the stereoselective microbial metabolic processes occurring in wastewater (see Step 3). As it is difficult to undertake *in vivo* metabolism studies of new abused drugs in humans, I tested if *in vitro* experiments utilising pHLM represented a valid and alternative method for metabolism investigation, especially for new designer drugs [31]. I therefore compared pHLM results with biological samples from animal samples (rat urine) and pooled human urine samples collected at festivals.

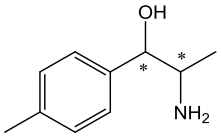
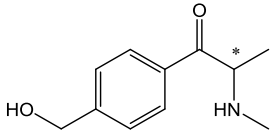
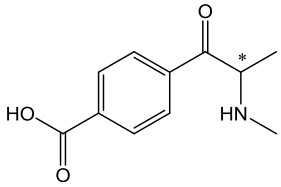
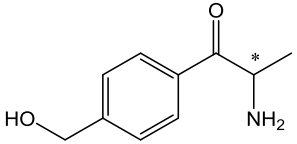
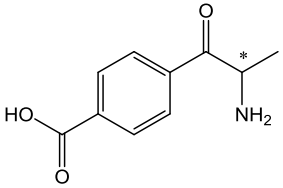
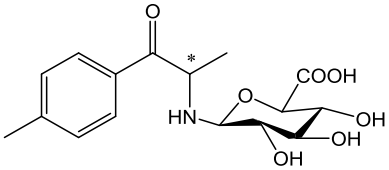
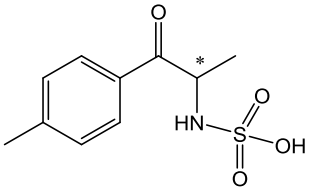
### 4.4.3 Step 3: Confirmation of metabolic residues in *in-vivo* and *in-vitro* studies

#### 4.4.3.1 Step 3(a): *In vitro* metabolism of mephedrone using pHLM

*In vitro* experiments were performed by incubating pHLM to verify the formation of phase I (experiment A in section 4.3.2.5) and phase II (experiment B in section 4.3.2.5) metabolites. Microsomes were previously used by Pedersen et al. [14] indicating the involvement of the isoenzyme CYP2D6 in the *in vitro* metabolism studies. In this study, the results obtained using chiral LC-TQD revealed a stereoselective metabolism of mephedrone leading to an enrichment with *R*-(+)-enantiomer (Figure 4-5) and formation of two metabolites: normephedrone and hydroxytolyl-mephedrone enriched with *S*-(-)-enantiomers.

**Table 4-1** Overview of mephedrone and its metabolites through target and non-target screening in all the samples investigated in this study.

| Analyte | Structures | Street mephedrone samples | Rat urine samples | Pooled urine samples | pHLM studies | Wastewater samples | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Influent for sampling campaign | WW incubated for stability study | WW with mephedrone incubation for the detection of mephedrone |
| Mephedrone |  | X | X | X | X[b], X[c] | X | X | X |
| Normephedrone |  | | X | | X[b], X[c] | | X | |
| 1-dihydro mephedrone |  | | | X | X[c] | X | | |
| Hydroxyl-mephedrone-3*O*-glucuronide |  | | X | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1-dihydro-normephedrone |  | | X | | | X | | |
| 4'-hydroxymethyl-mephedrone (hydroxytolyl-m.) |  | | X | | X[b] | X | | |
| 4'-carboxy-mephedrone |  | | X | | X[b] | X | | |
| Nor-hydroxytolyl-mephedrone [a] |  | | X | | | | | |
| 4'-carboxy-normephedrone[e] |  | | X | | X[c] | X | | |
| Normephedrone-*N*-glucuronide[d] |  | | | | X[c] | | | |
| *N*-normephedrone-sulphate [d] |  | | | | | X | | |

[a] Metabolites mentioned in Pedersen et al. 2013
[b] Metabolites producted by incubation of pHLM using set A experiment (Phase I)
[c] Metabolites producted by incubation of pHLM using set B experiment (Phase I and II)
[d] Metabolites not previously published
[e] Metabolites mentioned in Linhart et al. 2016

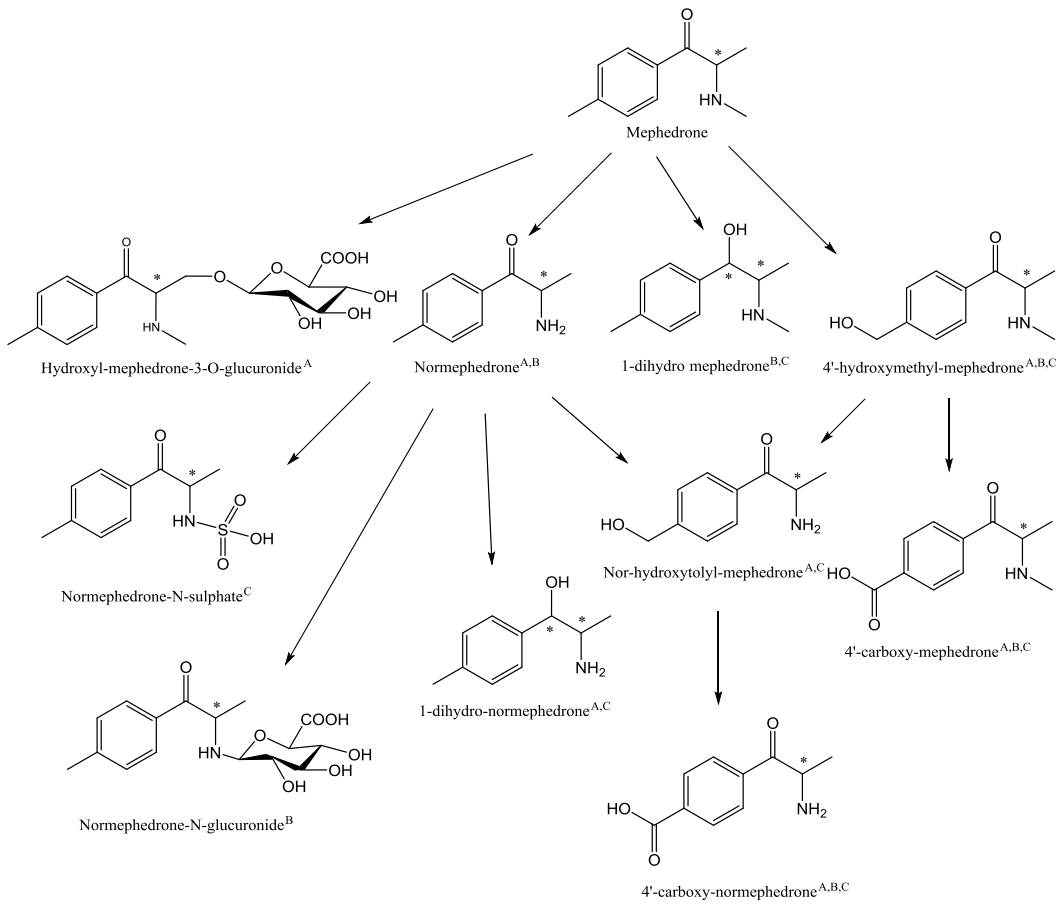



**Figure 4-3** (±)-Mephedrone metabolites detected in the investigated matrices and proposed scheme of metabolism ([A] Metabolites found in rat urine, [B] Metabolites found in pHLM study, [C] Metabolites found in wastewater).

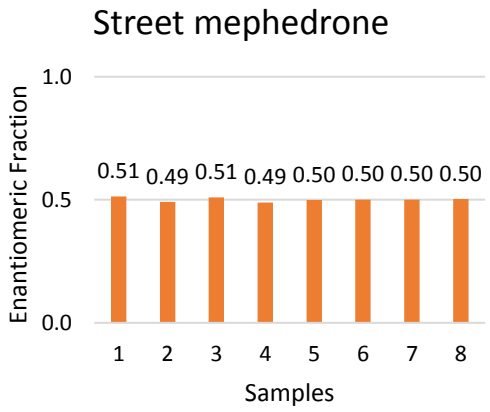



**Figure 4-4** Enantiomeric fraction of mephedrone in illegal samples of mephedrone. Results obtained by applying the unpaired t-test showed "t Stat < t Critical one-tail" (1.78 < 2.16), p two-tail (0.097) < 0.05. Therefore, EFs from street mephedrone samples were not significant different from EF=0.5 during validation. All t-tests and P-values can be found in Appendix 2 (S2).

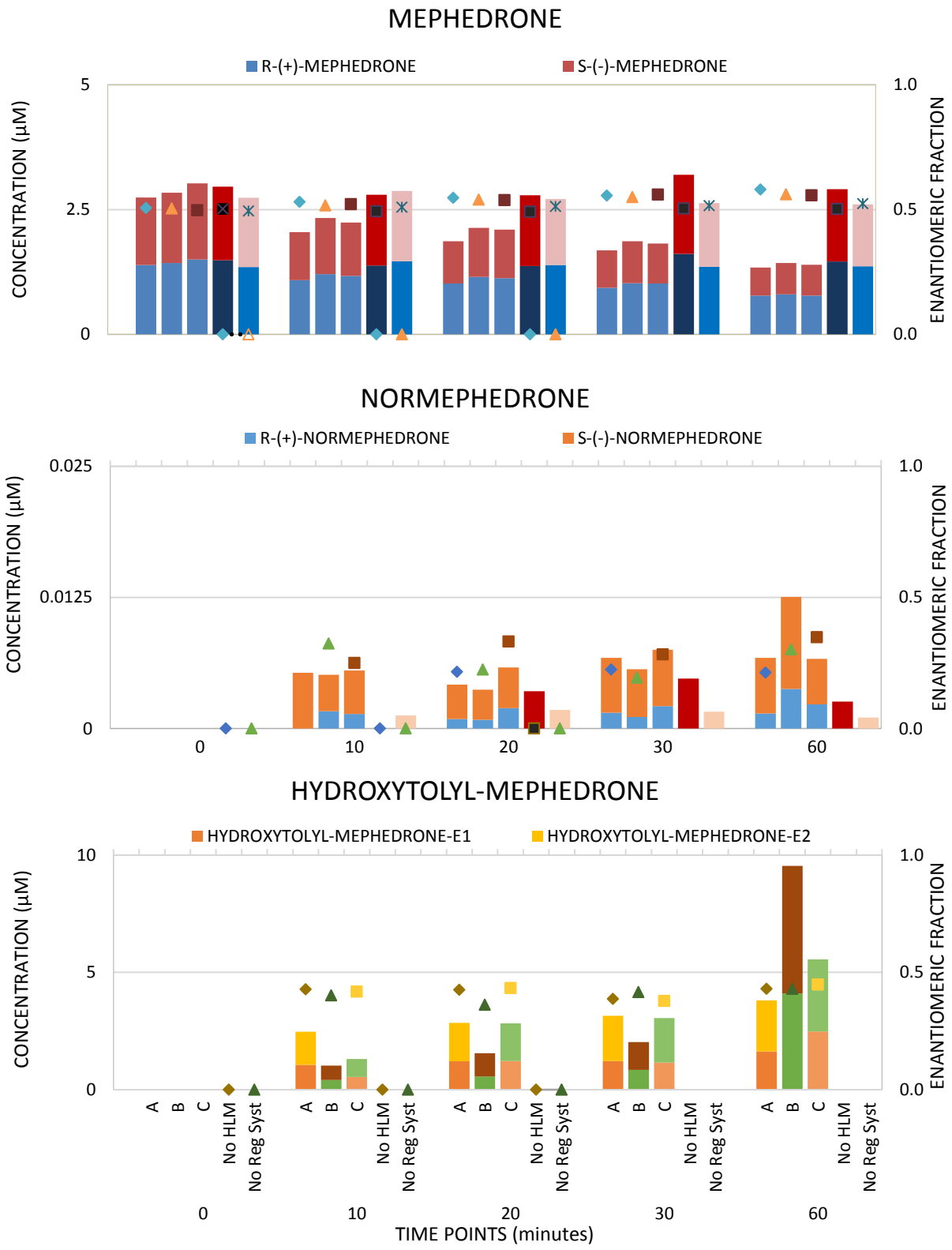



**Figure 4-5** Stereoselective metabolism of mephedrone and formation of metabolites in *in vitro* pHLM study. "A, B, C" are incubation reactors. All biological pHLM reactors are described in Table S2b.

LC QTOF non-targeted screening enabled the prediction and the detection of normephedrone and a new phase I metabolite, 4'-carboxy-normephedrone, by the use of MetID software. Dihydromephedrone, 4'-hydroxymethyl-mephedrone, 4'-carboxy-mephedrone and a new phase II metabolite normephedrone-*N*-glucuronide were predicted with a mass error <10 ppm for the precursor (for this reason they were not displayed in this paper) (Table 4-1 and S7, Figure 4-3). Regarding the latter metabolite, glucuronic acid was found to be conjugated to normephedrone *N*- group, in contrast to the 3*O*-glucuronide observed in Pozo et al. 2015 [21]. Target screening analysis by using LC-QTOF confirmed the presence of normephedrone (Table S8).

#### 4.4.3.2 Step 3(b): *In-vivo* metabolism of mephedrone in rat urine

The following mephedrone metabolites were detected in rat urine samples using LC Q-E (Table S10): normephedrone, hydroxy-tolyl-mephedrone, 4'-carboxy-mephedrone, 4'-carboxy-normephedrone, hydroxyl-mephedrone-3*O*-glucuronide. Chiral LC VP analysis of mephedrone excreted in the rat urine revealed that mephedrone undergoes stereoselective metabolism.

Both mephedrone and formed metabolites were not racemic in excreted urine: normephedrone (Figure S6), hydroxytolyl-mephedrone (Figure S7) and nor-hydroxytolyl-mephedrone (Figure S8). Diastereoisomers of dihydro-normephedrone were also found (Figure S7) and a predominance of one diastereoisomer with respect to the other was observed in this study. Linhart et al. (2016) observed a ratio for *erythro*- and *threo*- dihydro-normephedrone of 3:1 [20]. This ratio might also be confirmed in this study by assuming the same eluting profile as described in [20], although their analytical standards were not available.

Indeed, EFs were 0.49 ± 0.0 and 0.47 ± 0.0 for mephedrone and normephedrone in spiked control rat urine, respectively, whilst a dramatic decrease in EF (0.44 ± 0.0 and 0.22 ± 0.0 for mephedrone and normephedrone respectively) was observed in positive rat urine (Figure 4-6).

This data shows stereoselective metabolism of both compounds favouring *S-(-)*-enantiomer. This is in contrast to pHLM studies and human pooled urine. This study indicates that mephedrone metabolism in humans and rats might follow different stereoselective patterns.

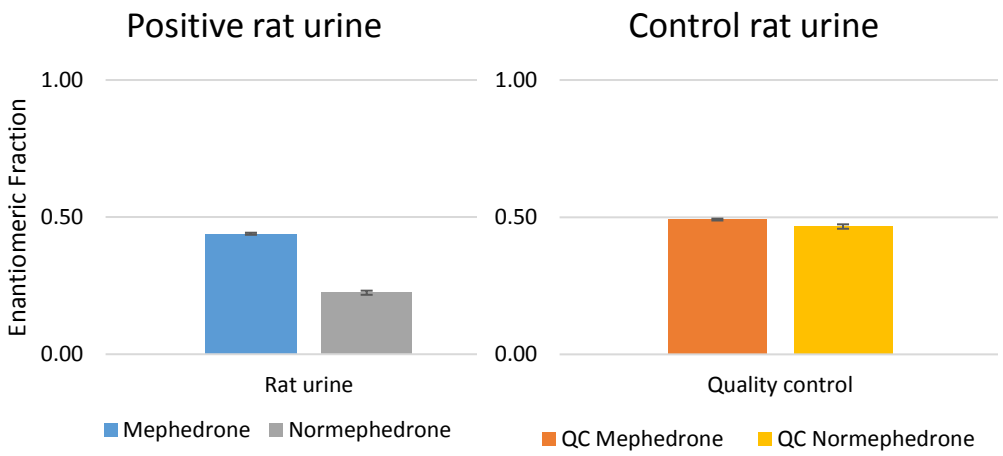



**Figure 4-6** Enantiomeric fraction of mephedrone in' positive' and in 'control' rat urine samples.

A possible hypothesis for this finding might be the hydroxylation reaction of *R-(+)*-normephedrone which lead to enrichment of mephedrone with *S-(-)*-normephedrone enantiomer. This also indicates that rat metabolism studies might not be indicative for human biomarker selection in WBE.

It is interesting to note that in the case of nor-hydroxytolyl-mephedrone, although this molecule has one chiral centre, four peaks showing the same fragmentation patterns were found (Figure S8). It was therefore hypothesised that the metabolic hydroxylation reaction may have occurred at different sites of the molecule. For this purpose, acetylated rat urine was injected in LC VP and GC-MS systems for suspected acetylated metabolites. Due to the presence of co-eluting interferences with some matrix compound, no distinguishable peaks for the enantiomers were found. No spectra corresponding to the (1-2 acetyl) hydroxyl-nor-mephedrone were found. Further work needs to be undertaken to explain this phenomenon.

#### 4.4.3.3 Step 3(c): Mephedrone in pooled urine

Chiral LC TQD analysis of pooled human urine samples confirmed predominance of *R*-(+)-mephedrone (Figure 4-7), hence its stereoselective metabolism. This is in agreement with wastewater samples and the pHLM study, indicating that mephedrone in wastewater resulted from its consumption on most days.

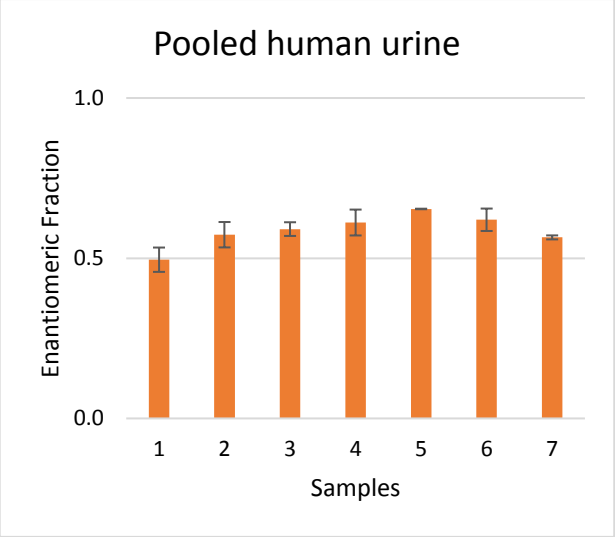



**Figure 4-7** Enantiomeric fraction of mephedrone in pooled human urine samples. Results obtained by applying the unpaired t-test showed "t Stat > t Critical one-tail" (4.88 > 1.89 for α=0.05; 4.88 > 4.78 for α=0.001), p one-tail (0.00089) < 0.001. Therefore, EFs from pooled human urine samples were significant different from EF=0.5. All t-tests and P-values can be found in Appendix 2 (S2).

Non-targeted screening with LC-QTOF and LC Q-E confirmed the presence of several metabolites that we found in wastewater, rat urine and in pHLM samples. These are: 4'-carboxy-mephedrone, 4'-carboxy-normephedrone, 1-dihydro-mephedrone (not in urine), 1-dihydro-normephedrone and hydroxyl-tolyl-normephedrone (not in pHLM). Interestingly, normephedrone was not detected in wastewater and in pooled urine samples.

### 4.4.4 Step 4: Microbial degradation in wastewater and verification of stability of possible biomarkers of mephedrone in wastewater

Incubation of mephedrone in wastewater over a duration of 24h did not lead to degradation and formation of by-products. Wastewater spiked with mephedrone was also incubated in a week long experiment. No formation of by-products was observed.

Stability of DTRs in wastewater is critical if low uncertainty measurements of community-wide drug abuse using WBE are to be undertaken. Good biomarkers need to be stable for at least 24 hours (so to ensure stability during 24-h composite sampling time). As recommended by the consensus best practise protocol for sampling and storage in Castiglioni et al. 2014 [13], low temperature settings reduce the degradation of biomarkers and help the preservation of the analytes in the samples. Indeed, our results after incubation of wastewater at differing experimental conditions (see section 4.3.2.4) confirmed that low temperature (4$^{o}$C)

reduces degradation of mephedrone with only approximately 10% change at 4°C when compared to up to 50% change at 17°C (Table 4-2, Figure S9). Furthermore, no formation of new metabolites was observed, which confirms stability of mephedrone in wastewater over a time of 24h at 4°C. In contrast, normephedrone was found to degrade at temperature settings.

**Table 4-2** Stability of targeted compounds in influent wastewater samples stored over a 48 hours.

| Analyte | Stability[a] [%] | | | | | |
|---|---|---|---|---|---|---|
| | Raw (unfiltered) wastewater, pH 7.4, stored at 17◦C | | | Raw (unfiltered) wastewater, pH 7.4, stored at 4◦C | | |
| | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h |
| *R*-(+)-Mephedrone | -23.4 ± 2.7 | -29.3 ± 4.7 | -48.5 ± 5.0 | -9.5 ± 2.7 | -10.9 ± 3.5 | -10.1 ± 6.3 |
| *S*-(-)-Mephedrone | -7.1 ± 4.8 | -10.2 ± 7.0 | -34.4 ± 10.1 | -4.0 ± 4.0 | -11.9 ± 17.1 | -23.9 ± 7.1 |
| *R*-(+)-Normephedrone | -19.0 ± 13.6 | -48.2 ± 73.2 | -39.2 ± 10.2 | 18.0 ± 95.3 | -19.9 ± 25.7 | -41.7 ± 15.1 |
| *S*-(-)-Normephedrone | -6.9 ± 0.0 | 1.5 ± 5.1 | -56.1 ± 17.8 | -5.1 ± 45.0 | -11.1 ± 1.0 | -10.1 ± 4.2 |

[a] Expressed as difference in percentage from to time-point 0 ± SD

## 4.5 Conclusions

This chapter proposes a new investigative framework for the selection and validation of metabolic biomarkers of abused drugs (such as NPSs) with limited information on their human metabolism with the ultimate goal of their application in WBE to estimate community-wide drug use. Mephedrone was chosen as a target chemical due to its widespread abuse in the UK and common presence in wastewater but little understanding of its metabolic profile.

The developed framework consisted of four steps and resulted in the following conclusions:

Step 1: Identification of possible metabolic biomarkers of mephedrone present in wastewater using LC-HRMS (in-direct *in-vivo* study).

Several metabolites of mephedrone and potential metabolic biomarkers were identified in wastewater. These are: dihydro-mephedrone, *N*-sulfo-normephedrone, hydroxy-tolyl-normephedrone, 4'-carboxy mephedrone, 4'-carboxy normephedrone, 1-dihydro-normephedrone.

Step 2: Verification of chiral signature of mephedrone using chiral LC-MS/MS.

'Street' mephedrone was found to be distributed in the UK as racemate.

Step 3: Confirmation of human metabolic residues in *in-vivo* (pooled urine) and *in-vitro* (pHLM) studies.

Stereoselective metabolism of mephedrone favouring *R*-(+)-enantiomer was observed in pHLM experiments and it was confirmed by pooled urine analysis. Interestingly, *in-vivo* rat metabolism studies led to contrasting results where *S*-(-)-mephedrone was favoured. This questions rat studies as a viable approach towards biomarker selection for WBE.

*In-vitro* pHLM experiments lead to identification of several metabolites and potential biomarkers of mephedrone abuse. These are: normephedrone, 4'-carboxy-normephedrone, dihydromephedrone, 4'-hydroxymethyl-mephedrone, 4'-carboxy-mephedrone, normephedrone-*N*-glucuronide. Interestingly, normephedrone formed via stereoselective metabolism of mephedrone in *in-vitro* pHLM studies was not identified in pooled urine samples. This might be due to dilution of pooled urine samples with urine from non-abusers.

Step 4: Microbial degradation in wastewater and verification of stability of possible biomarkers of mephedrone in wastewater.

Wastewater simulating microcosms revealed high stability of mephedrone with up to a week long incubation time at 4°C.

In the light of the above evidence, the following conclusions were drawn:

a. Mephedrone is a suitable candidate as a biomarker, because of its high stability in wastewater and stereoselective metabolism in humans.

b. Chiral analysis is fundamental in the enantiomeric profiling of mephedrone, especially in distinguishing between human consumption and direct disposal of unused drug.

c. Despite stereoselective formation of normephedrone in *in vitro* pHLM studies, this metabolite was found to be unsuitable as a biomarker of mephedrone consumption as (i) it was not detected in pooled human urine and in wastewater and (ii) due to its low stability in wastewater.

d. Possible biomarker candidates (apart from mephedrone) for future investigations are: 4'-carboxy-mephedrone, 4'-carboxy-normephedrone, 1-dihydro-mephedrone, 1-dihydro-normephedrone and hydroxyl-tolyl-normephedrone.

## 4.6 Contributions

Erika Castrignanò and Barbara Kasprzyk-Hordern planned and designed the study. Marie Mardal contributed to the experiments on the incubation wastewater study, pHLM study for phase I, rat urine analysis and their data analysis at the University of Saarland, Homburg, Germany. Markus R. Meyer supervised the experiments carried out at the University of Saarland, Homburg, Germany. At the University of Bath, Dr. G.Dan Pantos performed computational study and Axel Rydevik helped in interpreting LC-HRMS data. "Street" mephedrone samples (collected at Glastonbury Festival) and pooled urine samples were kindly provided by Bram Miserez, John Ramsey and Trevor Shine at TICTAC Communications Ltd, St. George's University of London, UK. Wastewater samples were provided by Wessex Water, UK.

## 4.7 Supplementary Data

The following supplementary data are contained in Appendix 2:

Figure S1 Synthesis of (±)-mephedrone (a, b) (modified from Schifano et al. (2010) [24]) and of *S*-(-)-mephedrone (c) (modified from Osorio-Olivares et al. (2003) [38]).

Figure S2 Proposed mephedrone metabolism in humans (modified from Pozo et al. (2014)).

Table S1 Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, a predicted using ACD/labs software (http://www.chemspider.com).

S1-Experimental settings and procedure for acetylation of rat urine sample.

Table S2 Experimental set up of the reactors used for (a) incubating wastewater and (b) pHLM.

Table S3 MRM transitions in chiral LC-TQD method.

Table S4 Method validation parameters (chiral LC-TQD) for mephedrone and normephedrone.

Table S5 Operating conditions for the absorbance and CD spectra of (±)-mephedrone (4-MMC)

Figure S3 CD and absorbance spectra of (±)-mephedrone (4-MMC) (a,b). UV spectra of (±)-mephedrone from the computational study (c).

Figure S4 Predicted CD spectra for (±)-mephedrone (a) and for (±)-normephedrone (b).

Table S6 Mephedrone concentrations and population-normalised mass loads in wastewater samples during one week monitoring campaign in 2014 and in 2015 in the UK.

Table S7 Non-targeted analysis by LC Q-TOF: mephedrone metabolites predicted in wastewater and in pHLM by using MetID software (Theor means theorethical and Exp. experimental).

Table S8 Target screening analysis in wastewater and in pHLM by using LC-QTOF.

Table S9 Untarget screening analysis in wastewater and in pHLM by using LC-QTOF.

Table S10 ddMS2 spectra of mephedrone metabolites detected in rat urine sample using LC Q-E.

Figure S6 MS2 chromatogram of mephedrone and normephedrone in rat urine sample using chiral LC VP.

Figure S7 Full scan chromatograms and spectra of mephedrone metabolites in rat urine sample using chiral LC VP. Dihydro-nor-mephedrone diastereoisomers are shown in (a), whilst the partial separation of hydroxytolyl-mephedrone enantiomers in (b).

Figure S8 Mephedrone metabolites in rat urine sample identified with chiral LC-VP: nor-hydroxytolyl-mephedrone and its two enantiomers (note: the presence of another minor mephedrone metabolite was observed).

Figure S9 Mephedrone and normephedrone concentrations and enantiomeric fraction in wastewater stability study. Picture of experimental settings.

## 4.8 References

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# Chapter 5: Enantiomeric profiling of illicit drugs in a pan-European study

## 5.1 Summary

The aim of this chapter is to present a first study on enantiomeric profiling of chiral drugs of abuse and potentially abused drugs in several European locations. WBE approach and chiral analysis were combined together, enabling the estimation of illicit drug usage at community level and the evaluation of their enantiomeric composition. This allowed extrapolating information on the nature of the drug residues by distinguishing between human consumption and direct disposal and on their synthesis routes.

Mephedrone was found in the UK with population-normalised mass loads up to 47.7 mg 1000 people$^{-1}$ day$^{-1}$, enriched with one enantiomer. This finding suggests a stereoselective metabolism in humans or stereoselective microbial metabolic processes occurring in the environment. A spatial trend was observed

between Northern and Southern European cities in the case of amphetamine loads with a slight enrichment of *R*-(-)-amphetamine in wastewater for the majority of sampling sites. Methamphetamine loads over the average were detected in Norway. This could be motivated by the high number of seizures occurred in this country as reported by the EMCDDA. The presence of the predominant *S*-(+)-methamphetamine enantiomer in wastewater samples from other European cities could be ascribed to its stereoselective synthesis in the illicit manufacturing market. The prevalence of *R*-(-)-MDMA in wastewater indicated that MDMA was present because of its consumption rather than its direct disposal. This was also observed in cities where previous monitoring campaigns revealed direct disposal of MDMA tablets. The enantiomeric profiling of MDMA showed an interesting metabolic pattern. In particular, *S*-(+)-MDA originated from MDMA metabolism rather than MDA itself, especially for MDA loads found during weekends. HMMA was more suitable as a MDMA biomarker than HMA, which was a good indicator of high abused MDMA levels only. Indeed, a slight enrichment of *S*-(+)-HMMA would mean MDMA abuse. Other biomarkers were also investigated such as precursors, MDEA, PMA, zopiclone, fluoxetine, norfluoxetine, venlafaxine, desmethylvenlafaxine and tramadol. The presence of precursors was reasonably ascribed to their medical use. MDEA and PMA were not found. Although high usage of zopiclone, fluoxetine and norfluoxetine were suspected, these drugs were lower than the method quantification limit (MQL). Venlafaxine: desmethylvenlafaxine ratio was 1:2 for the majority of countries. Controversial is the interpretation of the enantiomeric composition of both compounds due to different EF values. Very high tramadol loads were found in Brussels. Its chiral analysis showed an EF range between 0.53 and 0.61, indicating the predominance of the first eluting enantiomer.

## 5.2 Introduction

Since the first study by Zuccato et al. (2011) [1] on WBE to estimate community-wide illicit drug use trends, WBE has proven to provide valuable and complementary information to the traditional epidemiological strategies on public health [2, 3]. Indeed, the analysis of carefully selected biomarkers, which are often unique human urinary metabolic excretion products, allowed for near real-time

profiling of population health. Examples include the usage of illicit drugs [4, 5], legal highs [6, 7], alcohol [8] and tobacco [9].

WBE was utilised by several research groups to verify community-wide drug use [10-16]. The first European study in 2011 led by the Sewage Analysis CORe group Europe (SCORE) (www.score-cost.eu) involved 19 cities and estimated temporal and spatial drugs use trends across Europe [4]. This was followed by Europe-wide monitoring of 23 cities in 2012 [5] and then 42 cities in 2013 [17].There are several key stages in WBE: (i) biomarker selection; (ii) collection of representative wastewater samples; (iii) measurement of biomarkers in wastewater; (iv) calculation of the normalised-mass loads and, finally, (v) estimation of the drug consumption pro capita. Biomarker selection is considered to be of critical importance. This cannot be limited to the parent drug itself if determination of drug consumption estimate is the aim, since bias coming from disposal of unused drug might take place. A biomarker should be uniquely formed in the body (such as metabolite), be stable and present in wastewater at quantifiable concentrations. Unfortunately, as it is not always possible to select a unique metabolic biomarker, different solutions need to be sought. One of the innovative approaches focuses on enantiomerism of chiral drugs and their stereoselective metabolism.

Enantiomeric profiling can supplement WBE data with valuable information on abuse trends and potency of chiral drugs. It can also help with distinguishing between legal and illicit use of drugs as well as providing an indication of actual consumption as opposed to disposal of non-consumed drugs [2]. This is because drug synthesis is associated with different chiral signatures that depend on synthetic routes. Furthermore, chiral drugs undergo stereoselective disposition in humans leading to changes in the chiral signature (EF, enantiomeric fraction) [18]. For example, MDMA is produced as racemate but it undergoes stereoselective metabolism leading to enrichment of MDMA excreted in urine with *R*-(-)-enantiomer due to its longer elimination rate with respect to *S*-(+)-isomer [19]. The potential in using enantioselective analysis for WBE purposes was demonstrated in several studies, even though the main investigation of illicit drugs at enantiomeric level was focused on understanding the fate of chiral drugs during wastewater treatment and in the environment [20]. Kasprzyk-Hordern and Baker [19] confirmed that amphetamine found in wastewater in the UK was from illicit

use (and not from medical use) and that MDA was present in wastewater as a result of MDMA consumption rather than MDA use. Vázquez-Roig et al. reported usage patterns of chiral drugs in the catchment area of Valencia (Spain) [21], by linking selective enrichment of MDMA with *R*-(-)-enantiomer in wastewater to human consumption. Enantioselective analysis also helped in understanding that the unexpectedly high quantity of MDMA detected during a monitoring campaign in 2011 in Utrecht was due to direct disposal of unused MDMA as a consequence of a police raid at a nearby illegal production facility [22]. Similarly, Petrie et al. linked high levels of fluoxetine in wastewater with disposal of unused drug rather than its consumption [23]. Recently developed by Castrignanò et al., a multi-residue enantioselective method enabled simultaneous analysis of 56 biomarkers allowing for the first time the detection of mephedrone enantiomers in wastewater samples in the UK [24].

Despite these findings, a limited number of studies correlated the enantiomeric composition of chiral biomarkers to official statistics [20]. Hence, this is the first pan-European study aimed at investigating enantiomeric profiling of "common" drugs of abuse, new synthetic drugs and chiral pharmaceuticals in eight cities from eight different countries (i.e. a population equivalent of 4942979). The focus of this research was to:

- quantify selected drugs in wastewater from eight European locations,
- verify if drug residues in wastewater were originated from direct disposal of unused drug or their consumption,
- verify new emerging trends and any changes in drug usage across Europe.

## 5.3 Experimental

### 5.3.1 Chemicals and Materials

The following chiral analytes were selected in this study (Figure 5-1): (±)-mephedrone, (±)-4-hydroxy-3-methoxymethamphetamine (HMMA), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-4-hydroxy-3-methoxyamphetamine (HMA), (±)-methamphetamine, (±)-amphetamine, (±)-3,4-methylenedioxyamphetamine (MDA), (±)-tramadol, (±)-desmethylvenlafaxine, (±)-venlafaxine, (±)-3,4-methylenedioxy-*N*-ethyl-amphetamine (MDEA), (±)-ephedrine, (±)-pseudoephedrine, (±)-*para*-methoxyamphetamine (PMA), (±)-norephedrine, (±)-norfluoxetine, (±)-zopiclone, (±)-fluoxetine.

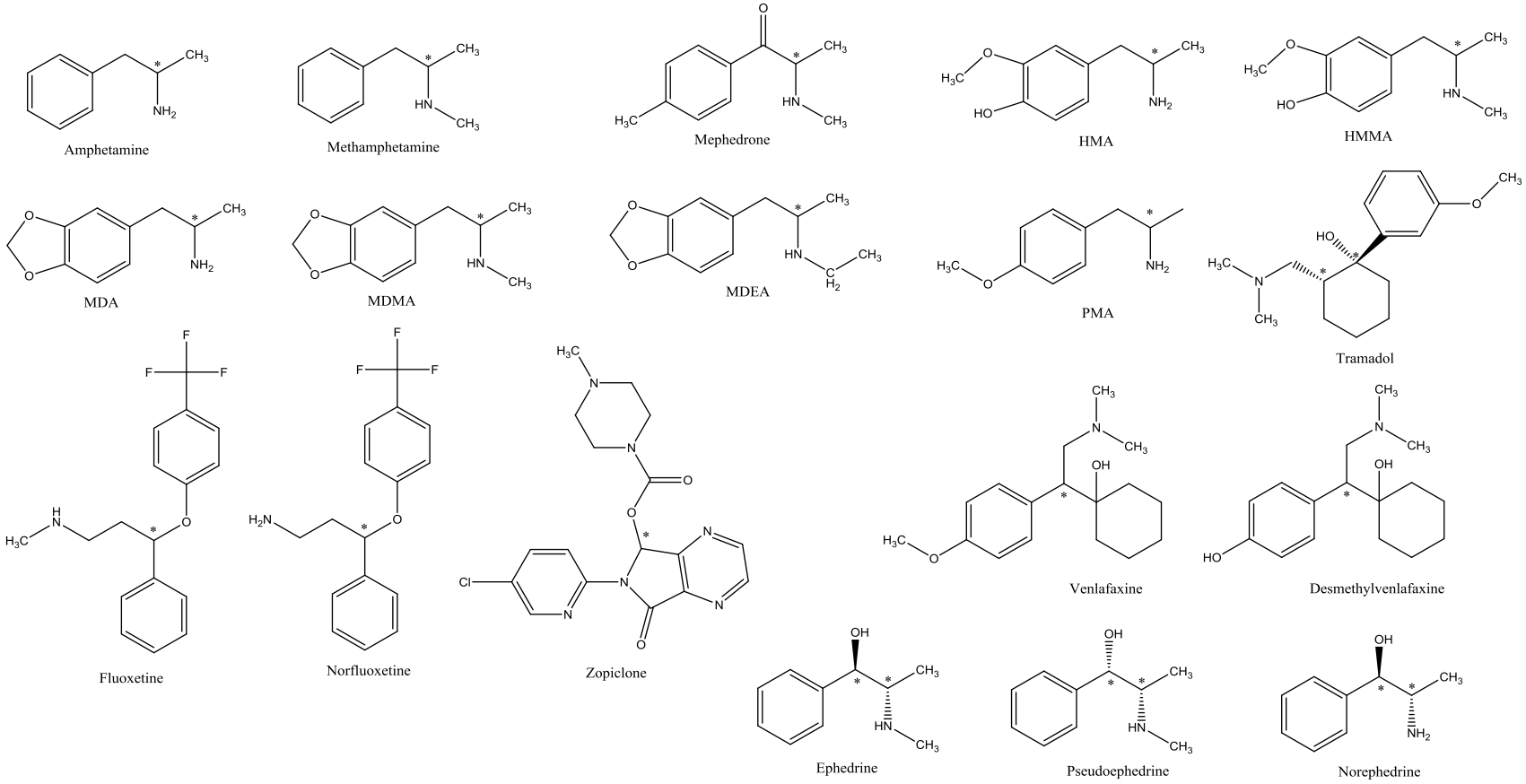



**Figure 5-1** Chiral analytes selected in the study.

Table S1 shows all analytes, their CAS number, molecular formula, molecular weight, $pK_a$ and supplier information. Amphetamine-$D_5$, methamphetamine-$D_5$, mephedrone-$D_3$, MDA-$D_5$, MDMA-$D_5$, MDEA-$D_5$, zopiclone-$D_4$ and *1S,2R*-(+)-ephedrine-$D_3$ were used as internal standards (IS). All standards and ISs were of the highest purity available (>97%). Stock and working solutions of standards were stored at -20° C. Methanol, acetonitrile and ammonium acetate were purchased from Sigma Aldrich, UK. Ultrapure water was obtained from PURELAB UHQ-PS Unit (Elga, UK). Deactivation of the glassware was carried out with 5% DMDCS followed by washing with toluene and methanol in order to prevent the adsorption of basic analytes to the hydroxyl sites on the glass surface.

### 5.3.2 Sample collection, storage and sample preparation

24-hours composite wastewater influent samples were collected over seven consecutive days in March 2015 from several wastewater treatment plants across Europe using best practice sampling protocol [25]. The week in March was chosen as a "routine week", in which no national and local festivities were taking place. Sampling sites were in Oslo (Norway), Bristol (United Kingdom), Lyngby (Denmark), Utrecht (The Netherlands), Brussels (Belgium), Zurich (Switzerland), Milan (Italy) and Castellon (Spain) (Figure 5-2).

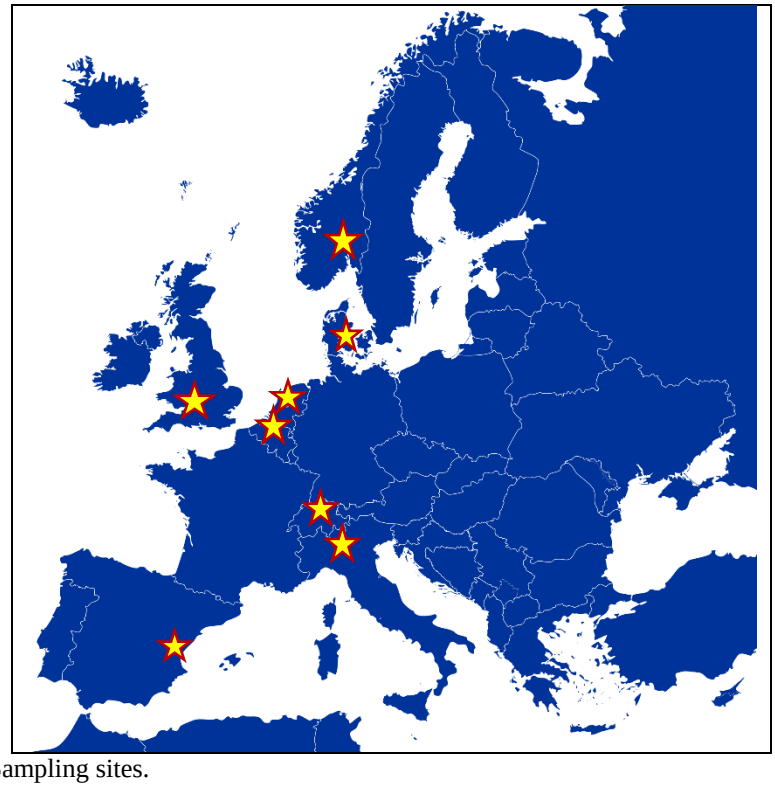


**Figure 5-2** Sampling sites.

Table 5-1 provides information on population and flow for the selected cities in the study. After collection, samples were transported to the local laboratory in refrigerated conditions and shipped on ice blocks to the UK within 24 hours. A fully validated analytical method was used for the detection and quantification of chiral drugs of abuse in wastewater as described elsewhere [24]. Briefly, the samples were filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK). 100 µL of IS mix (concentration 1 mg $L^{-1}$) were added to 100 mL of a wastewater sample.

**Table 5-1** Selected cities in the study, population and flow data.

| **City** | Bristol | Oslo | Milan | Utrecht | Castellon | Brussels | Zurich | Copenhagen |
|---|---|---|---|---|---|---|---|---|
| **Country** | UK | Norway | Italy | The Netherlands | Spain | Belgium | Switzerland | Denmark |
| **Residential population** | 886650 | 580639 | 1100000 | 300000 | 180690 | 954000 | 410000 | 531000 |
| **Day** | **Flow in $m^3$/day** | | | | | | | |
| Monday | 197493.3 | 254570.5 | 597470.0 | 45970.0 | 37469.0 | 234774.0 | 177167.0 | 148724.0 |
| Tuesday | 204490.8 | 252721.5 | 423110.0 | 44580.0 | 40476.0 | 359951.0 | 160912.0 | 150936.0 |
| Wednesday | 198950.4 | 333480.1 | 403240.0 | 47740.0 | 50228.0 | 234264.0 | 157084.0 | 147175.0 |
| Thursday | 197523.0 | 308279.1 | 412310.0 | 45030.0 | 49161.0 | 235442.0 | 161005.0 | 144840.0 |
| Friday | 252682.2 | 277449.7 | 402240.0 | 49530.0 | 43728.0 | 234906.0 | 161427.0 | 145197.0 |
| Saturday | 220687.2 | 256766.4 | 403020.0 | 46030.0 | 38301.0 | 233096.0 | 200010.0 | 137793.0 |
| Sunday | 193194.0 | 250383.9 | 422690.0 | 46900.0 | 37243.0 | 230375.0 | 243013.0 | 137244.0 |

SPE was carried out using Oasis HLB cartridges (60 mg, Waters, UK) and the following procedure. The cartridges were conditioned with 2 mL of methanol followed by equilibration with 2 mL of ultrapure water at a rate of 3 mL $min^{-1}$. The wastewater sample was passed through the HLB cartridge at a rate of 8 mL $min^{-1}$. The cartridges were then washed with 3 mL of ultrapure water at a rate of 3 mL $min^{-1}$ and the analytes were eluted with 4 mL of methanol at a rate of 8 mL $min^{-1}$ into 5 mL silanised glass tubes. The extract was transferred to the TurboVap evaporator (Caliper, UK). After evaporation to dryness under nitrogen flow (5-10 psi) at 40 °C the samples were reconstituted with 0.5 mL 1mM ammonium acetate/methanol 85:15 v/v and filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm). The filtered samples were transferred to polypropylene plastic vials bonded pre-slit PTFE/Silicone septa (Waters, UK) and then 20 µL were directly injected into a chiral HPLC-MS/MS.

### 5.3.3 Sample analysis

Samples were analysed in triplicate using HPLC-MS/MS system. Separation of all chiral analytes was undertaken with a CHIRALPAK® CBH HPLC Column 5 µm particle size, L × I.D. 10 cm × 2.0 mm (Chiral Technologies, France) with a chiral-CBH guard column 10 × 2.0 mm, 5 µm particle size (Chiral Technologies, France) using a Waters ACQUITY UPLC® system (Waters, Manchester, UK) under isocratic conditions at a 0.1 mL min-1. The mobile phase was made of a solution 1 mM ammonium acetate/methanol 85:15 v/v. The temperature was kept at 4 °C in the ACQUITY UPLC™ autosampler, whilst at 25 °C in the column compartment. The injection volume was set at 20 µL.

A triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an electrospray ionisation source was used in positive mode operating in the multiple reaction monitoring (MRM) mode. Table S2 shows MRM transitions used for selected analytes. MassLynx 4.1 (Waters, UK) was used to control the Waters ACQUITY system and the Xevo TQD. Data processing was carried out using TargetLynx software (Waters, Manchester, UK). Method validation data are provided in Tables S3-S7.

### 5.3.4 Calculations

Enantiomeric fractions (EF) were calculated using equation (7) reported in chapter 3. In order to obtain daily mass loads, the concentrations of analytes expressed in ng $L^{-1}$ were multiplied by the flow rate (L $day^{-1}$) and then normalised by the population size of the catchment area. This was essential for comparing data coming from different cities involved in the study. Figure 5-3 shows the trend of the population-normalised loads and the EF along the monitoring week, whilst Figure 5-4 shows the average weekly population- normalised loads and their weekly average EF. Estimated community-wide consumption was calculated using population-normalised mass loads and correction factors for excretion pattern (CF) of 3.3, 2.3 and 1.5 for amphetamine, methamphetamine and MDMA respectively [26, 27]. Estimated data for amphetamine, methamphetamine and MDMA usage are shown in Tables S8-S10.

## 5.4 Results and Discussions

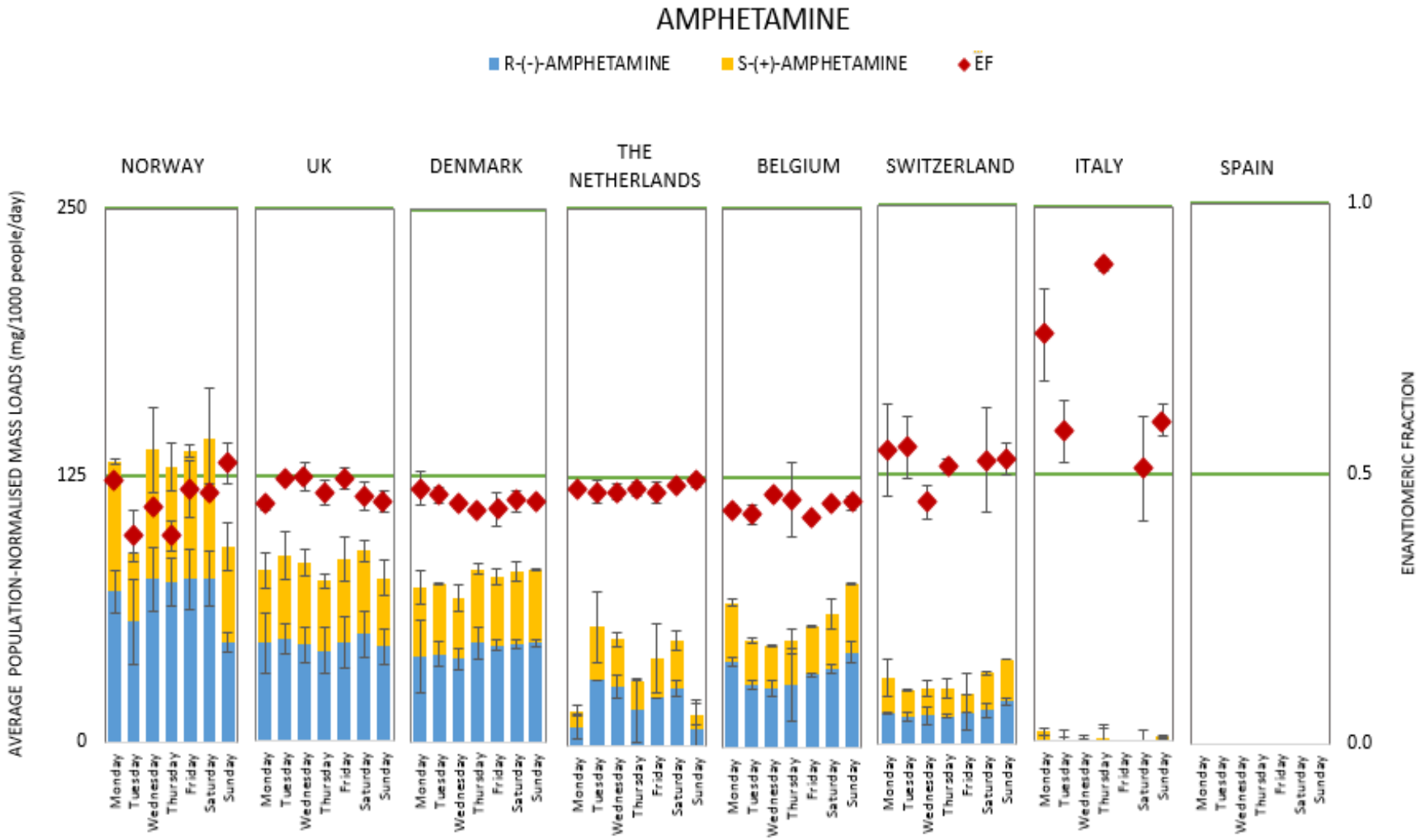
For simplicity, in the graphics results are referred and shown with the fullnames of the countries (and not with those of the cities).

### 5.4.1 Amphetamine and Methamphetamine

Data on amphetamines consumption reported by the European drug report 2015 showed that 1.3 million of Europeans within a range age of 15 - 34 years old used amphetamines in the last year [28]. This data was obtained by EMCDDA's five key epidemiological indicators, which consist of "estimates of recreational use (based mainly on surveys), estimates of high-risk use, drug-related deaths, infectious diseases and drug treatment entry" along with Reitox focal points and other sources [28]. In this work, we applied WBE approach to estimate amphetamine and methamphetamine use in eight European cities. Unfortunately, no metabolic biomarkers of amphetamine and methamphetamine exist that can be reliably used to estimate their abuse via WBE. Therefore, amphetamine and methamphetamine itself are commonly used as biomarkers. This constitutes a problem as the analysis of parent drugs does not usually allow for distinction if (meth)amphetamine residue present in wastewater comes from direct disposal of unused drug or its consumption.

Additionally, amphetamine is also a metabolite of other (prescription) drugs, such as fenethylline, fenproporex, methamphetamine [29] and selegiline [5]. Moreover, the percentage of the unchanged amphetamine fraction in urine can change due to changes in urine pH: at neutral pH 30% of amphetamine is found unchanged in urine within 24 hours [29], acid urine may contain up to 74% of amphetamine, while alkaline urine only 1%. This could lead to high uncertainty of calculations and possible over or underestimation of amphetamine use.

The awareness of this uncertainty is well recognised in the scientific community studying amphetamine abuse through WBE [30], [31], [32], [26]. As reported by Ort et al. (2014) [5], the evaluation of an effective consumption of amphetamine has to be carried out along with methamphetamine data to distinguish between drug consumption from its metabolism. However, as in the case of amphetamine, methamphetamine excretion is pH of urine dependent. For example, at pH 6-8, the unchanged methamphetamine is present at 43% in urine in 24 h, while its pharmacologically active metabolite amphetamine at only 4-7%. At acidic pH, up to 76% of unchanged methamphetamine can be excreted in urine in 24h.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ITALY
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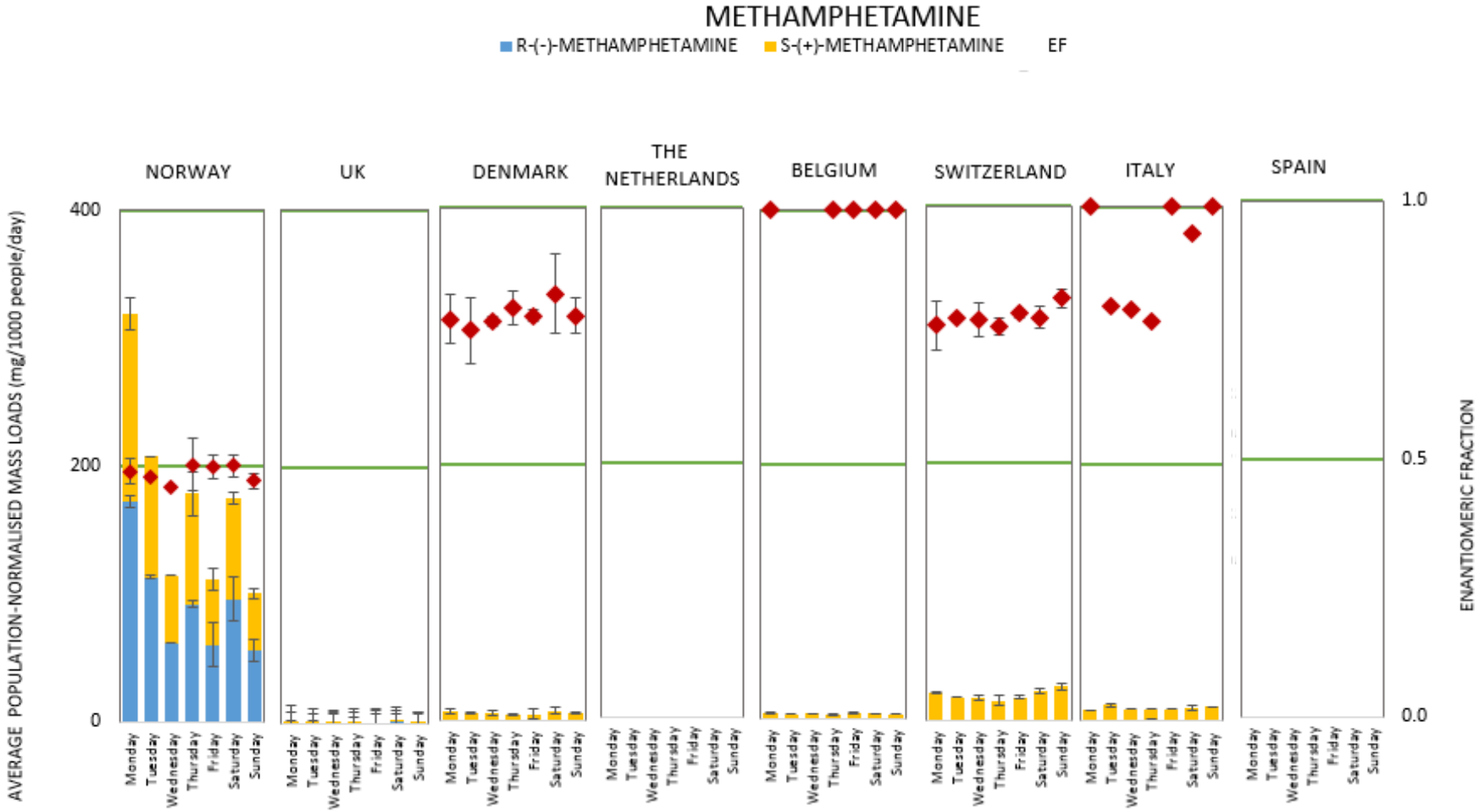
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AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
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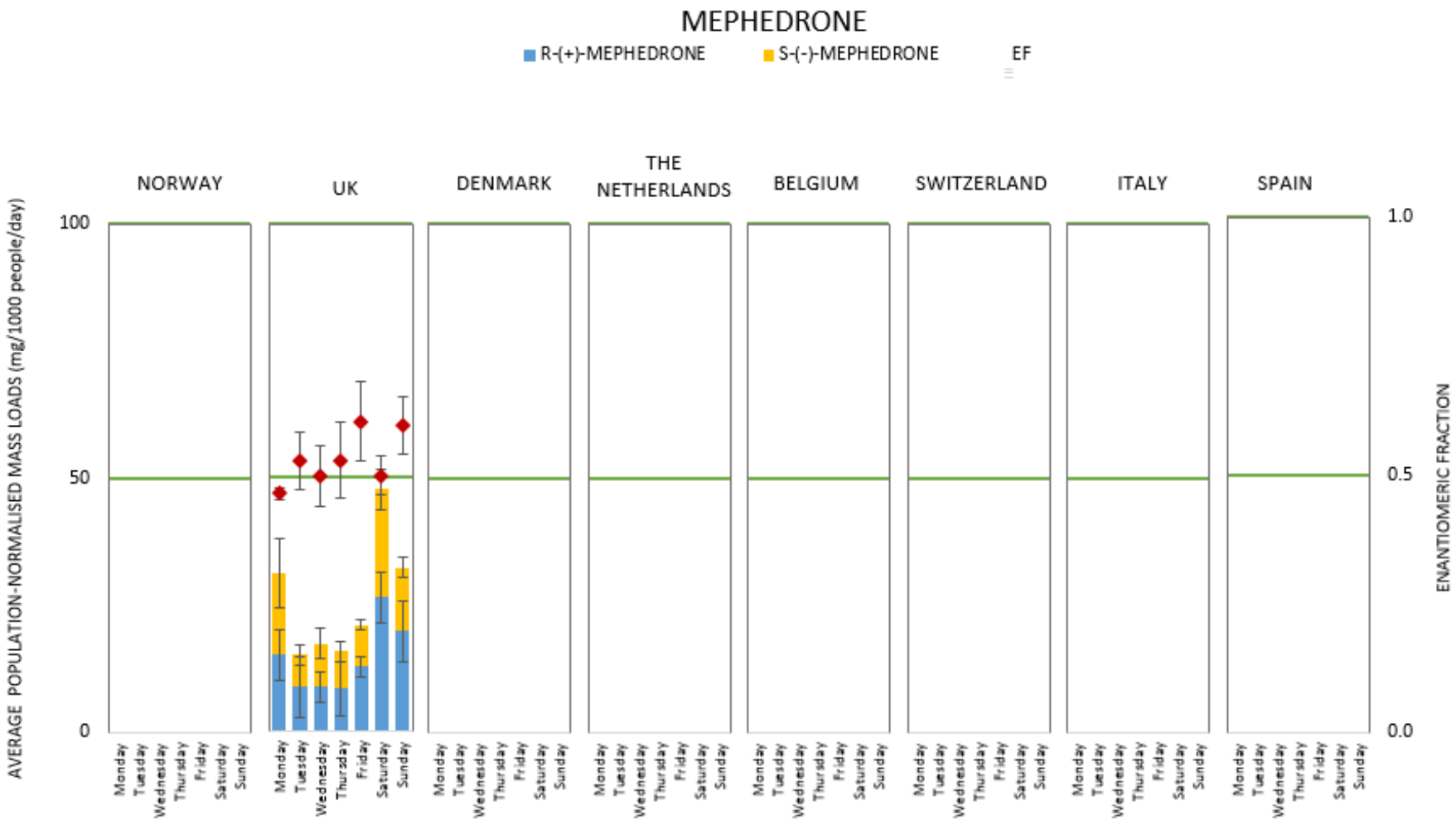
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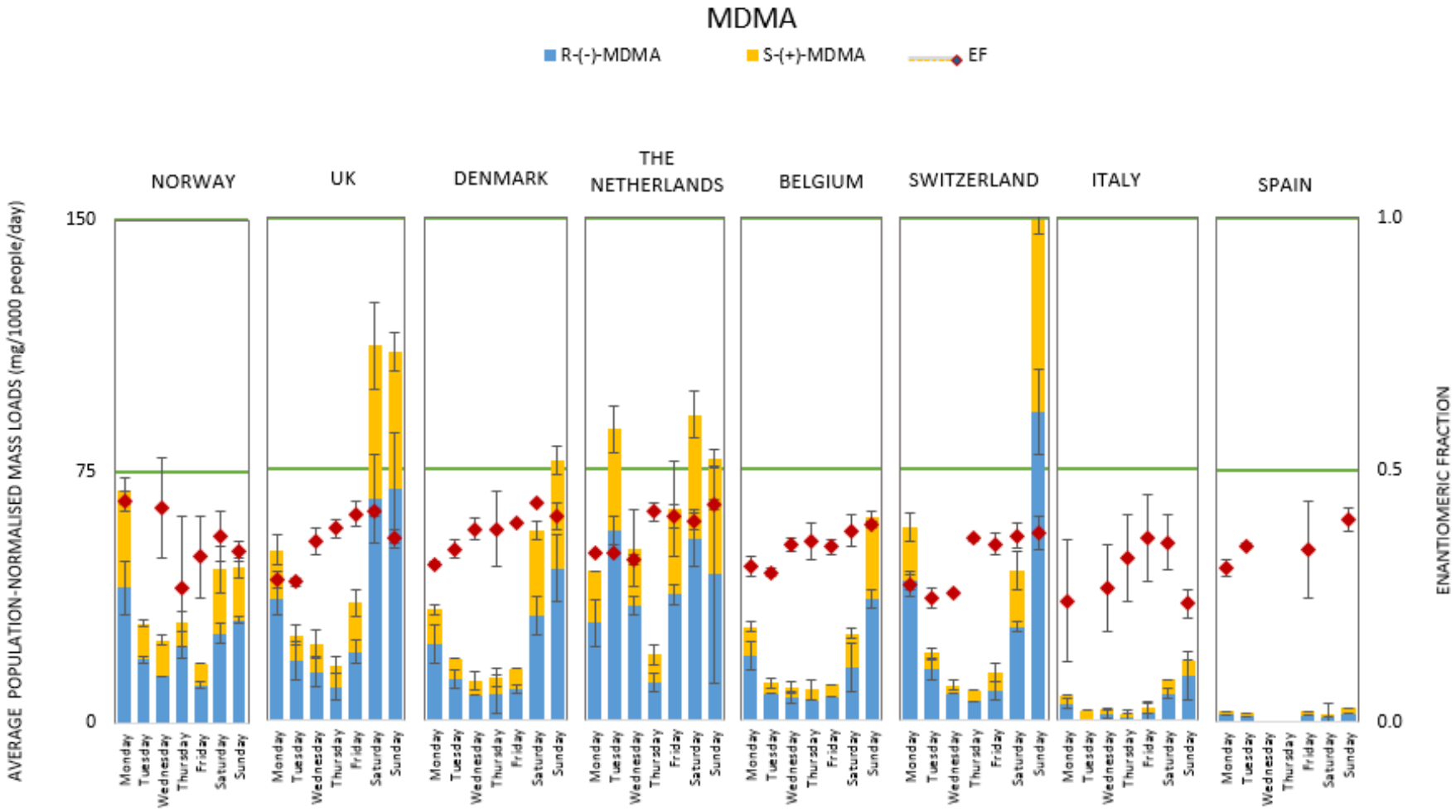
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AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
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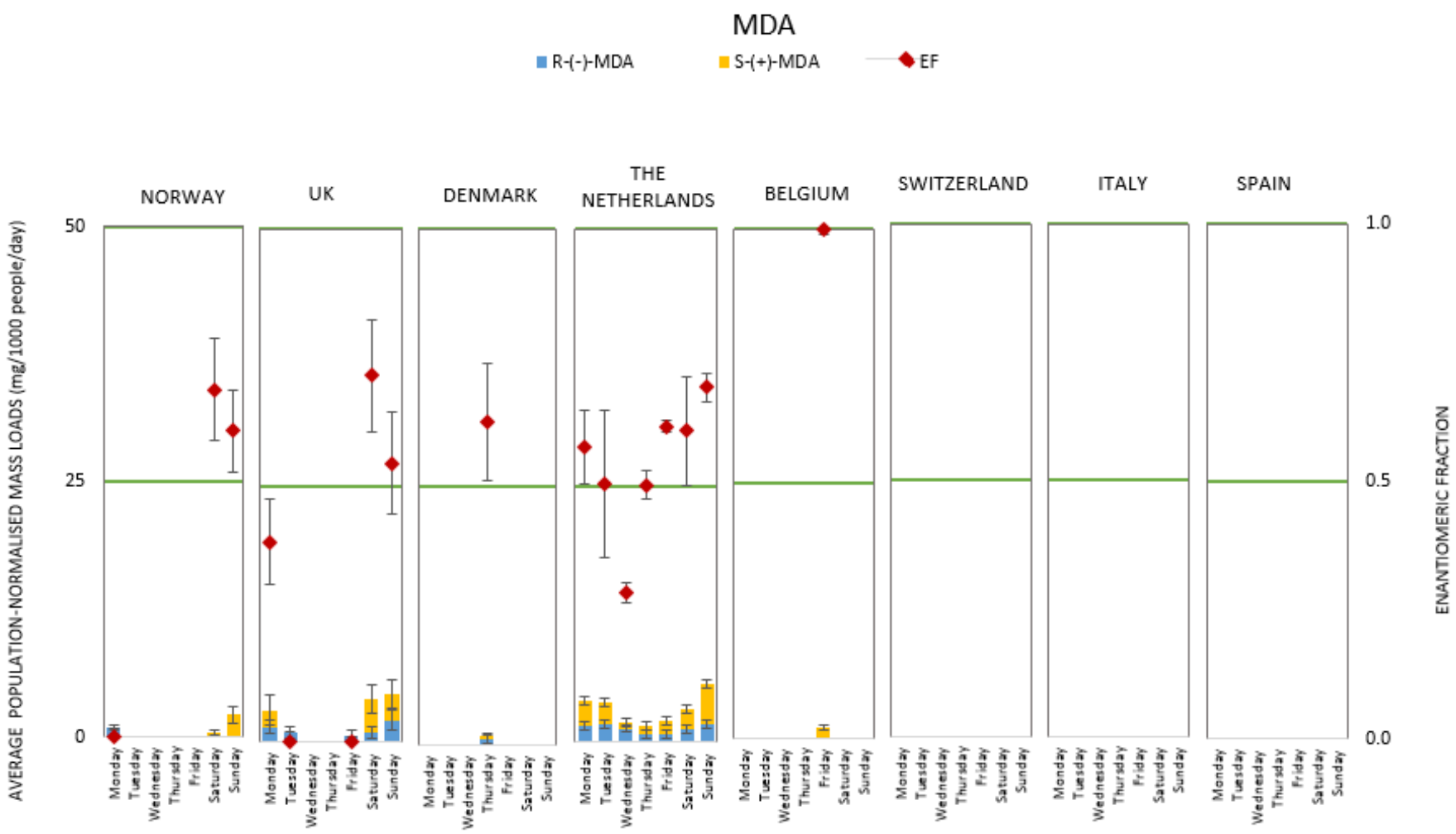
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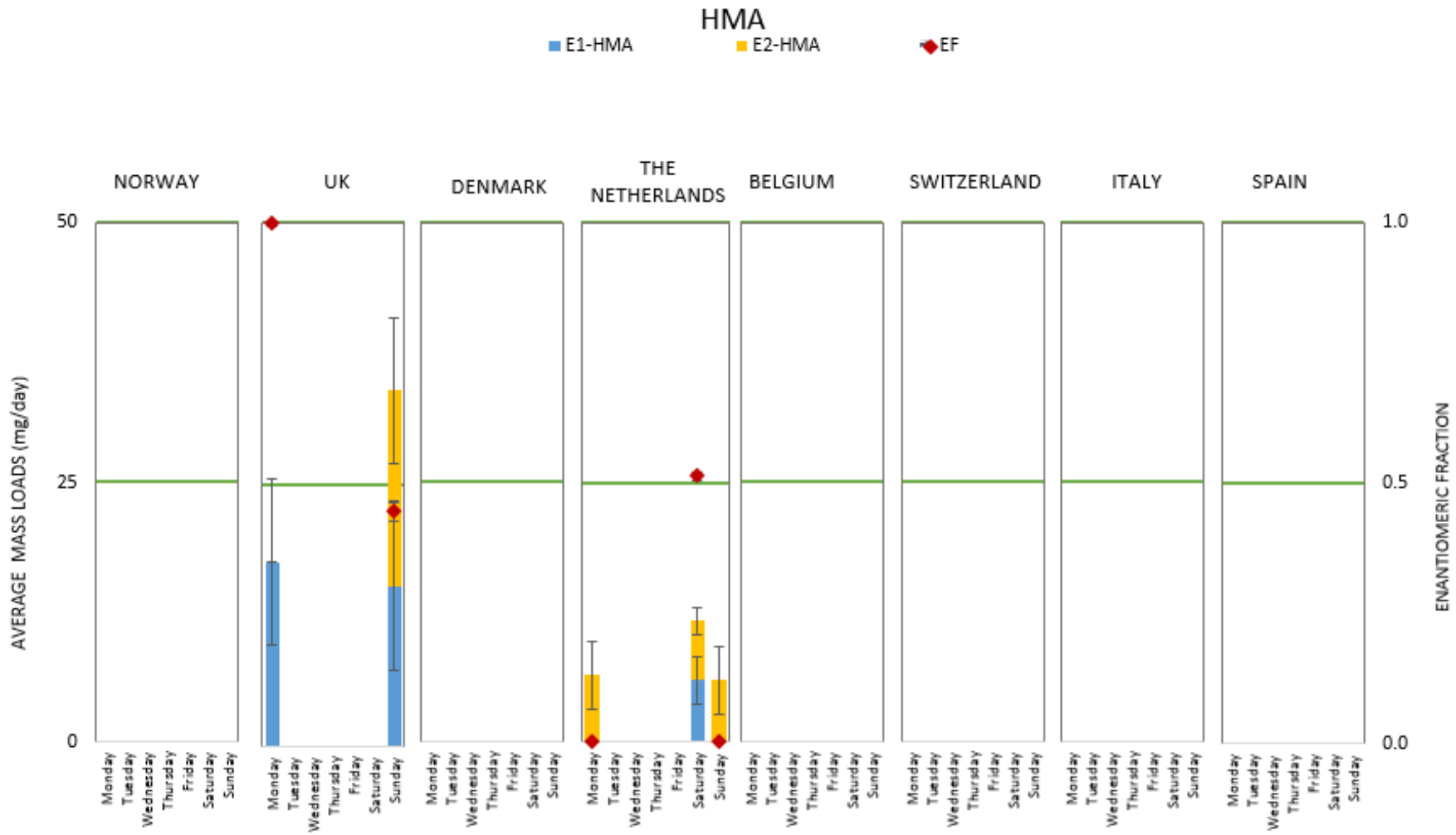
HMA
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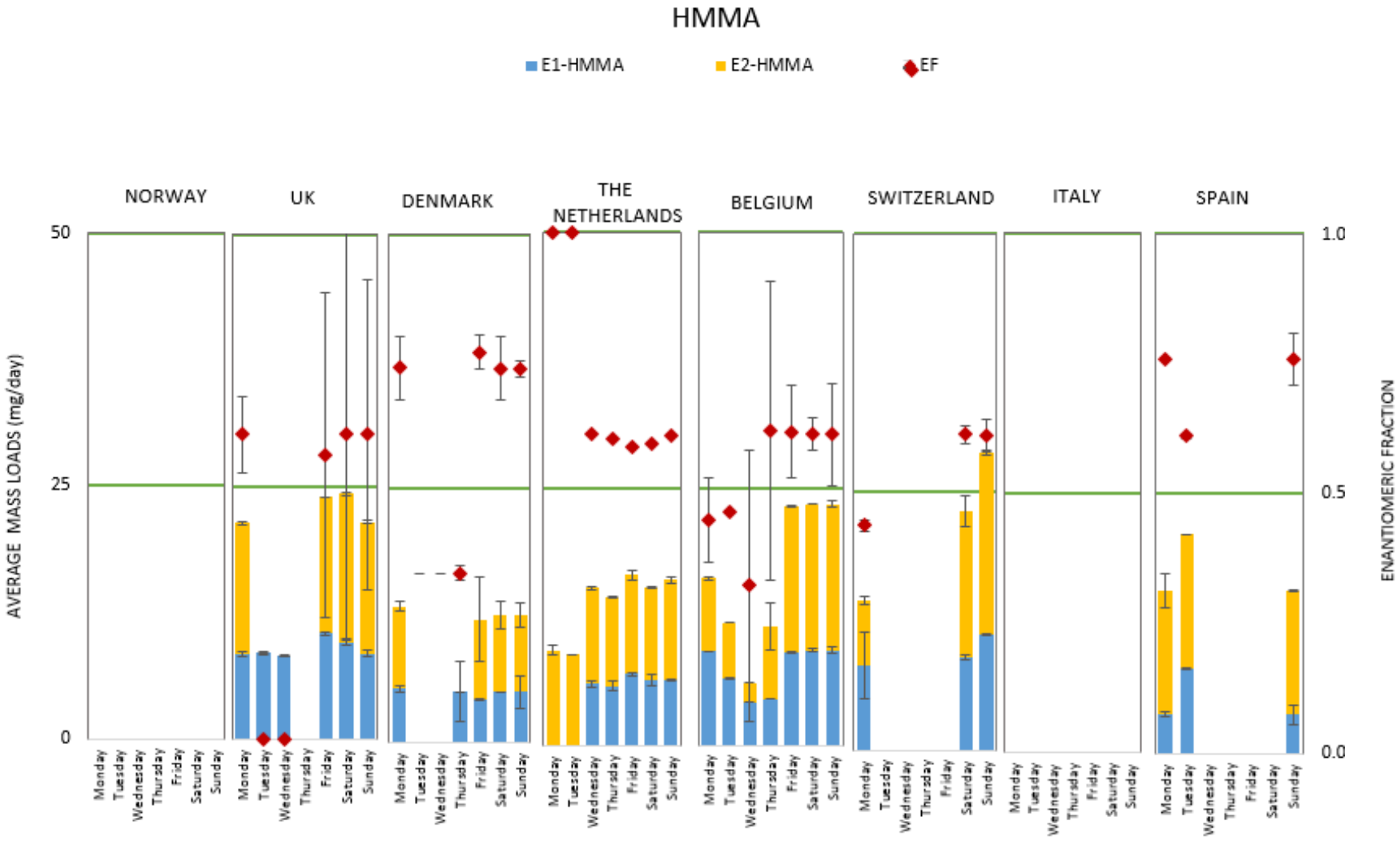
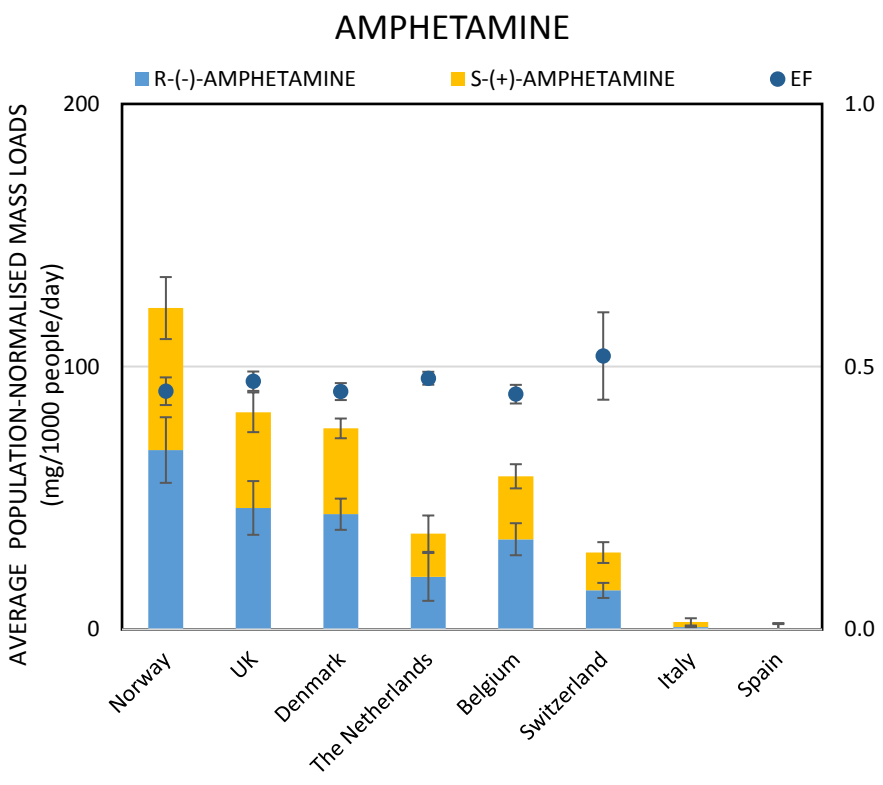
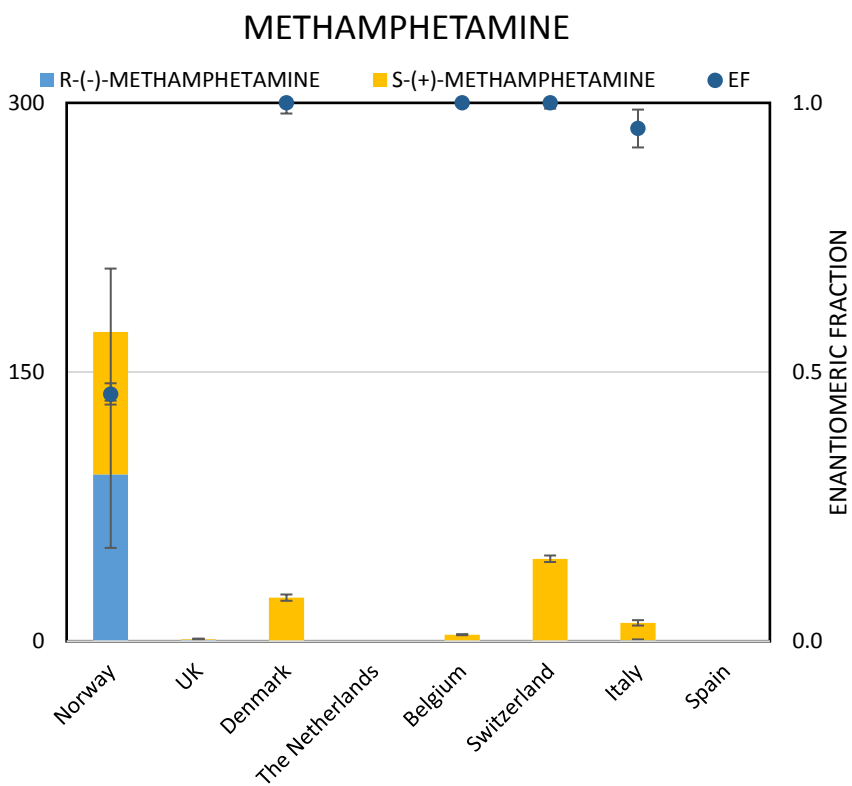


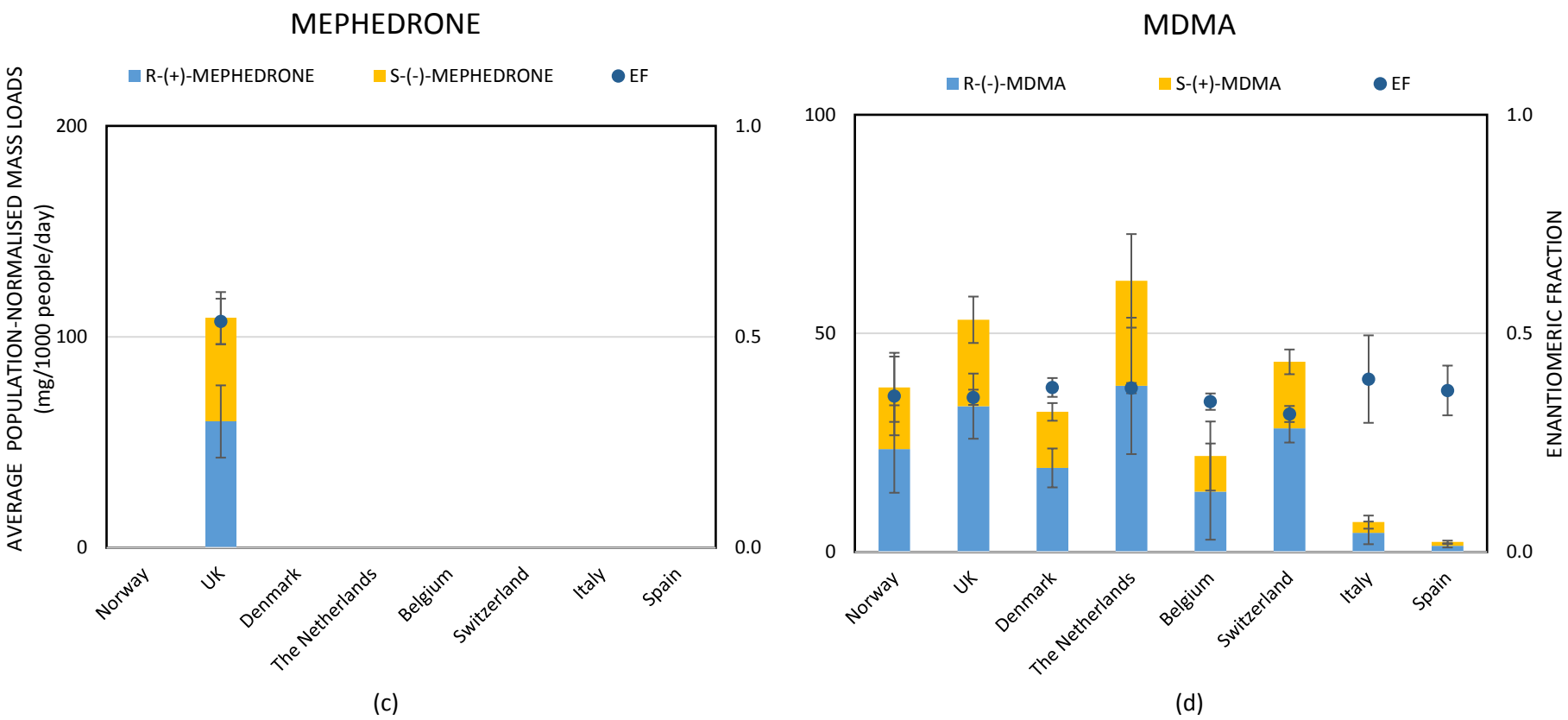

**Figure 5-3** Population-normalised mass loads and enantiomeric fraction values in a week monitoring campaign. Here, results are shown with the fullnames of the countries (and not with those of the cities). For HMMA: EF values displayed in the graphics are reported using the equation E2/(E1+E2), assuming that E1 is *R*-(-)-HMMA and E2 is *S*-(+)-HMMA.

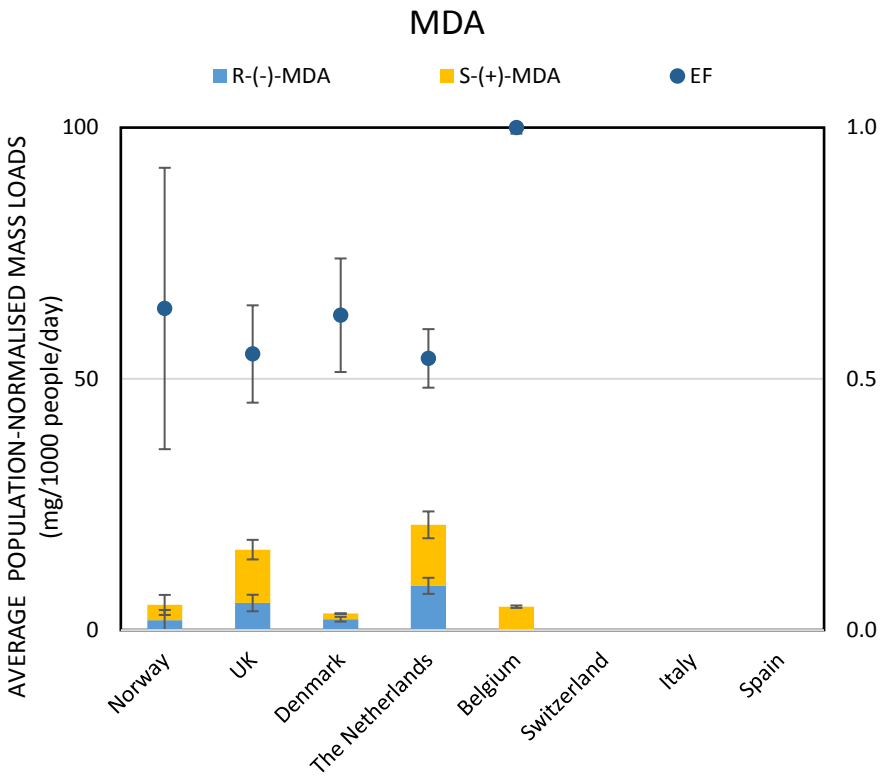

AMPHETAMINE
R-(-)-AMPHETAMINE
S-(+)-AMPHETAMINE
EF
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
200
100
0
1.0
0.5
0.0
Norway
UK
Denmark
The Netherlands
Belgium
Switzerland
Italy
Spain

(a)

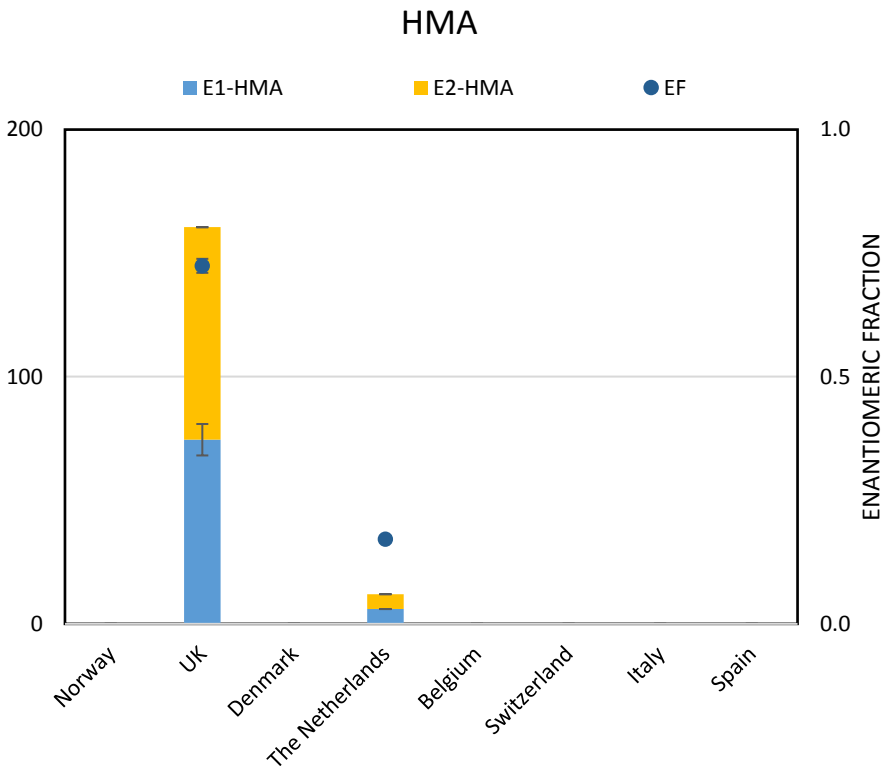

METHAMPHETAMINE
R-(-)-METHAMPHETAMINE
S-(+)-METHAMPHETAMINE
EF
300
150
0
1.0
0.5
0.0
ENANTIOMERIC FRACTION
Norway
UK
Denmark
The Netherlands
Belgium
Switzerland
Italy
Spain

(b)

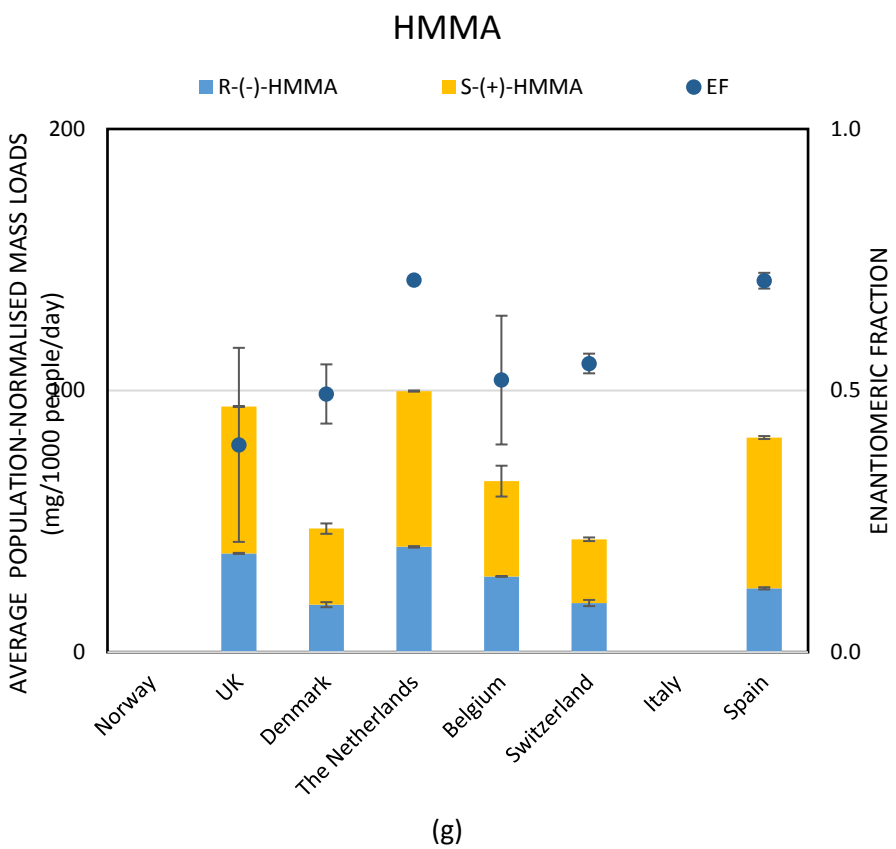

MEPHEDRONE
R-(+)-MEPHEDRONE
S-(-)-MEPHEDRONE
EF
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
200
100
0
1.0
0.5
0.0
Norway
UK
Denmark
The Netherlands
Belgium
Switzerland
Italy
Spain
(c)
MDMA
R-(-)-MDMA
S-(+)-MDMA
EF
100
50
0
1.0
0.5
0.0
ENANTIOMERIC FRACTION
Norway
UK
Denmark
The Netherlands
Belgium
Switzerland
Italy
Spain
(d)


MDA
R-(-)-MDA
S-(+)-MDA
EF
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
100
50
0
1.0
0.5
0.0
Norway
UK
Denmark
The Netherlands
Belgium
Switzerland
Italy
Spain

(e)


HMA
E1-HMA
E2-HMA
EF
200
100
0
1.0
0.5
0.0
ENANTIOMERIC FRACTION
Norway
UK
Denmark
The Netherlands
Belgium
Switzerland
Italy
Spain

(f)




(g)

**Figure 5-4** Average population-normalised mass loads and average enantiomeric fraction values in a week monitoring campaign. Here, results are shown with the fullnames of the countries (and not with those of the cities). For HMMA: EF values displayed in the graphic are reported using the equation E2/(E1+E2), assuming that E1 is *R*-(-)-HMMA and E2 is *S*-(+)-HMMA. Statistical evaluation obtained by applying the unpaired t-test to amphetamine showed that "t Stat > t Critical one-tail" for all wastewater samples excluding Switzerland (8.25 > 1.81 for $\alpha$=0.05, 8.25 > 4.14 for $\alpha$=0.001), p one-tail < 0.001. Therefore, EFs from wastewater samples were significant different (EF<0.5) from EF=0.5 during validation. All t-tests and P-values can be found in Appendix 3 (S1).

On the other hand, at alkaline urine only around 2% of methamphetamine will be excreted [33]. Furthermore, verification of amphetamine/methamphetamine ratio cannot provide comprehensive information on drug consumption against direct disposal of unused drug. Additional evidence is therefore needed to distinguish between amphetamine abuse from its direct disposal or its formation as a metabolite of other drugs. The phenomenon of enantiomerism of amphetamines may contribute in helping with it.

Amphetamine has one chiral centre and exists in two enantiomeric forms. Amphetamine's enantiomers have different activity: *S*-(+)-amphetamine is more potent than the *R*-(-)-enantiomer in eliciting Central Nervous System (CNS) effects, whilst *R*-(-)-amphetamine in cardiovascular effects [34]. Synthesis of racemic amphetamine is commonly performed via the Leuckart method, which uses 1-phenyl-2-propanone, formic acid, ammonium formate or formamide as reagents [35]. A stereoselective method, which involves the reduction of appropriate diastereoisomers of norephedrine or norpseudoephedrine [36], is much less common [37]. In Europe, licit amphetamine is prescribed as enantiomerically pure *S*-(+)-amphetamine formulations (e.g. dexamfetamine sulphate known with trade name Dexamed, Attentin, Tentin) in the UK, Denmark, Finland, Ireland, Luxembourg, the Netherlands, Norway, Spain, and Sweden [38] and as racemate in prescriptions drugs only under the Medicines Act. The prodrug lisdexamfetamine in the form of dimesylate salt is completely metabolised to *S*-(+)-amphetamine and it is also available in the European market [39, 40]. Pharmaceuticals, such as fenproporex [41] and clobenzorex [42], are metabolised to 25-35% and 5% of racemic amphetamine, respectively [43]. If excreted as metabolite of selegiline, which intake is only *R*-(-)-enantiomer, it is enriched with *R*-(-)-amphetamine along with *R*-(-)-methamphetamine [44]. Amphetamine undergoes enantioselective metabolism by favouring *S*-(+)-enantiomer (elimination half-life of *S*-(+)-amphetamine is slightly shorter than *R*-(-)-amphetamine [45]) and leading to enrichment of amphetamine with *R*-(-)-enantiomer when excreted in urine. Therefore, if racemic amphetamine is consumed, it will be found in wastewater enriched with *R*-(-)-enantiomer.

Similarly to amphetamine, methamphetamine enantiomers are known to have different activity. The *S*-(+)-enantiomer has a central stimulant activity. It is used in the treatment of obesity and it is also abused as illicit drug. The *R*-(-)-

enantiomer has a prominent peripheral sympathomimetic activity and for this reason it is used as a nasal decongestant in non-prescription inhalers in the American market [29]. On the illicit market, methamphetamine is synthesised in two ways. The first starts from the reactions of phenylacetone (i.e. the Leuckart route and reductive amination) giving a racemic mixture of methamphetamine, whilst the other starts from (1*R*,2*S*)-(-)-ephedrine [or (1*S*,2*S*)-(+)-pseudoephedrine] that is reduced with red phosphorus and hydriodic acid giving a stereoselective synthesis of the *S*-(+)-isomer. EMCDDA reports indicate that methamphetamine commonly found in Europe is primarily enantio-enriched [46]. However, the synthesis with phenylacetone as precursor is preferred in Lithuania. Similarly to amphetamine, methamphetamine undergoes stereoselective metabolism in humans by favouring *S*-(+)-enantiomer [43] and leading to the enrichment of methamphetamine in urine with *R*-(-)-enantiomer with a changing enantiomeric ratio over the time. Controlled pharmaceuticals, such as mefenorex, produce racemic methamphetamine as metabolite, whilst others containing famprofazone as active compound are converted to 30% *S*-(+)-methamphetamine and 70% *R*-(-)-enantiomer [43]. Therefore, if racemic methamphetamine is consumed, it will be found in urine enriched with *R*-(-)-enantiomer. Additionally, generated amphetamine will be enriched with *S*-(+)-enantiomer. If enantiomerically pure *S*-(+)-methamphetamine is consumed, it will be excreted with urine as enantiomerically pure *S*-(+)-methamphetamine and enantiomerically pure *S*-(+)-amphetamine. This is because chiral inversion does not take place during human metabolism of methamphetamine.

In this study population normalised mass-loads of amphetamine ranged from <MQL in Castellon to a maximum weekly average value of 122.3 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo. In particular, the weekly average values were the following:

- 122.3 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo (twice as low amount was reported in 2013 [17]) with loads below 100 mg $day^{-1}$ 1000 $people^{-1}$ only in two days over a week-sampling campaign. *R*-(-)-enantiomer was mainly predominant across the monitoring week (EF<0.5) with an exception on Sunday in which EF was 0.52.
- 82.6 mg $day^{-1}$ 1000 $people^{-1}$ in Bristol (exactly the same amount was reported in 2014 [17]) with a higher value only on Saturday of 89.6 mg $day^{-1}$ 1000 $people^{-1}$ with a predominance of *R*-(-)-enantiomer. EF values were lower than 0.5 apart for three days in which EF were about 0.5 (Tuesday, Wednesday and Friday).

- 76.5 mg day$^{-1}$ 1000 people$^{-1}$ in Lyngby (lower amount was found in 2013 in Copenhagen [17]) with higher loads during the weekend and an EF average value lower than 0.5 for all the week.
- 36.4 mg day$^{-1}$ 1000 people$^{-1}$ in Utrecht (46.5 and 111.1 mg day$^{-1}$ 1000 people$^{-1}$ [17] were reported in 2013 and in 2014 respectively) with higher loads from Tuesday to Saturday. EF values were always just slightly below 0.5.
- 58.3 mg day$^{-1}$ 1000 people$^{-1}$ in Brussels (24.8 mg day$^{-1}$ 1000 people$^{-1}$ was reported in 2013 [17]) with a clear weekend trend and EF values always lower than 0.5.
- 29.3 mg day$^{-1}$ 1000 people$^{-1}$ in Zurich (42.7 and 25.7 mg day$^{-1}$ 1000 people$^{-1}$ were reported in 2013 and in 2014 respectively [17]) with a slight tendency in higher loads in the weekend. The average EF was 0.5, but fluctuating values above and below 0.5 were found throughout the week.
- 2.9 mg day$^{-1}$ 1000 people$^{-1}$ in Milan (values were below limit of quantification (LOQ) since 2012 [17]) with no trend observed during the weekend. EF values were reported even though high error bars are displayed due to low concentrations.

The average population-normalised amphetamine loads showed a decreasing amphetamine usage from Northern to Southern cities. Oslo shows higher amphetamine prevalence among Northern European cities. Indeed, only Italian and Spanish cities were notably below the overall mean loads 34-28 mg day$^{-1}$ 1000 people$^{-1}$ reported in the 2013 European study [5] (the first value is referred to the cities participating in all 3 years study, the second to all cities participating in the corresponding year). Temporal trends show that amphetamine loads increased in Oslo, Lyngby, Brussels and Milan, even if they are very low for the latter city. They remained stable in Bristol and decreased in Zurich and in Utrecht.

However, results on amphetamine are in line with previous monitoring studies undertaken in years 2012-15 [5]. Furthermore, enantiomeric profiling revealed that amphetamine in wastewater is enriched with *R*-(-)-enantiomer in most European cities. This could indicate the consumption of racemic amphetamine. Interestingly, amphetamine was found to be enriched with *S*-(+)-enantiomer in Zurich and Milan. This suggests either usage of *S*-(+)-amphetamine (prescribed or illicit) or its formation as a result of metabolism of methamphetamine. This is very likely as

methamphetamine in both cities was found to be enriched with *S*-(+)-enantiomer (see discussion below).

Population normalised mass-loads of methamphetamine ranged from <MQL in Utrecht and Castellon to a maximum value of 172.4 mg day$^{-1}$ 1000 people$^{-1}$ in Oslo. According to the EMCDDA Report [28], high methamphetamine seizures were seen in Norway and this could explain the high loads detected. In details, the results were the follows:

- 172.4 mg day$^{-1}$ 1000 people$^{-1}$ as weekly average value in Oslo. This value is definitely lower than 237.4 mg day$^{-1}$ 1000 people$^{-1}$ reported in 2014 but still higher than 107.9 in 2013 [17]. The highest amount was found on Monday (318.5 mg day$^{-1}$ 1000 people$^{-1}$) and EF values were close or below 0.5.
- 1.2 mg day$^{-1}$ 1000 people$^{-1}$ in Bristol (a similar amount was reported in 2014 [17]). EF values were not displayed because of the proximity of the concentrations to the LOQ.
- Weekly average 6.6 mg day$^{-1}$ 1000 people$^{-1}$ in Lyngby (<LOQ and 9.8 mg day$^{-1}$ 1000 people$^{-1}$ were reported for the Danish capital respectively in 2014 and in 2013 [17]). Due to the presence of only *S*-(+)-enantiomer in the samples, EF values were equal to 1. The loads were quite uniform during all the week.
- 3.7 mg day$^{-1}$ 1000 people$^{-1}$ as weekly average value in Brussels (1.7 mg day$^{-1}$ 1000 people$^{-1}$ was reported in 2013 [17]) with a very steady trend in the weekend. EF values were always equal to 1 because of the presence of one enantiomer only.
- 20.2 mg day$^{-1}$ 1000 people$^{-1}$ as weekly average value in Zurich (16.7 and 21.8 mg day$^{-1}$ 1000 people$^{-1}$ were reported in 2013 and in 2014 [17]) with a slight tendency in higher loads in the weekend. S-(+)-enantiomer was the only enantiomer found, so the average EF was 1.
- 10.3 mg day$^{-1}$ 1000 people$^{-1}$ in Milan (values were 5.9 and 5.4 mg day$^{-1}$ 1000 people$^{-1}$in 2013 and 2014 respectively [17]).

The overall mean value ranged from 17 to 33 mg day$^{-1}$ 1000 people$^{-1}$ in 2013 [5] (as before, the first value is referred to the cities participating in all 3 years study, the second to all cities participating in the corresponding year). Zurich was found to have the second high methamphetamine loads of 20.2 mg day$^{-1}$ 1000 people$^{-1}$ as weekly average. Estimates in Lyngby and Brussels were below the overall mean value. Other European locations showed low levels. Despite being below the European average, Italian data showed doubled amount of methamphetamine load

when compared to data from the same area in 2013-14 and reaching 2012 loads [17].

Enantiomeric profiling of European wastewater samples revealed that methamphetamine distributed in most European locations is enantiomerically pure of *S*-(+)-methamphetamine. Norwegian wastewaters were an exception as they contained either racemic methamphetamine, which indicated direct disposal of unused racemic methamphetamine, or methamphetamine enriched with *R*-(-)-enantiomer, which indicated consumption of racemic methamphetamine. This data can be confirmed by the fact that racemic methamphetamine, produced in Lithuania, is exported to Norway (and Sweden). Therefore, this synthetic route is different from that one observed in Central Europe. Interestingly, as *S*-(+)-methamphetamine is the most potent psychotropic enantiomer [47] than racemic methamphetamine, one can conclude that despite lower usage of methamphetamine in Zurich, Lyngby, Brussels and Milan, potency of the drug is much higher than in Oslo.

### 5.4.2 MDMA and MDA

Data on ecstasy consumption reported by the European drug report 2015 assessed that 1.8 million of Europeans with a range age from 15 and 34 years old used ecstasy in the last year, with a low and stable prevalence trends [28]. MDMA is the main ingredient of ecstasy tablets. It was suggested that there is an increased availability of high-content MDMA tablets, powder and crystals in Europe [28].

Europe-wide MDMA usage was also estimated using WBE [4, 5]. Unfortunately, estimations are based on quantification of MDMA as a DTR in wastewater. Such an approach does not allow for accurate evaluation of MDMA consumption against the direct disposal of unused drug. There are two possible solutions: (1) specific metabolic biomarkers should be sought as MDMA is known to extensively metabolise to MDA, DHMA and HMMA (Figure 5-5) and (2) enantiomeric profiling should be implemented as MDMA undergoes stereoselective metabolism leading to the formation of chiral metabolites.

MDMA has one chiral centre and as a result it exists in two enantiomeric forms. MDMA enantiomers have different activity: *S*-(+)-enantiomers are more amphetamine-like stimulants and *R*-(-)-enantiomers are more hallucinogenic [48].

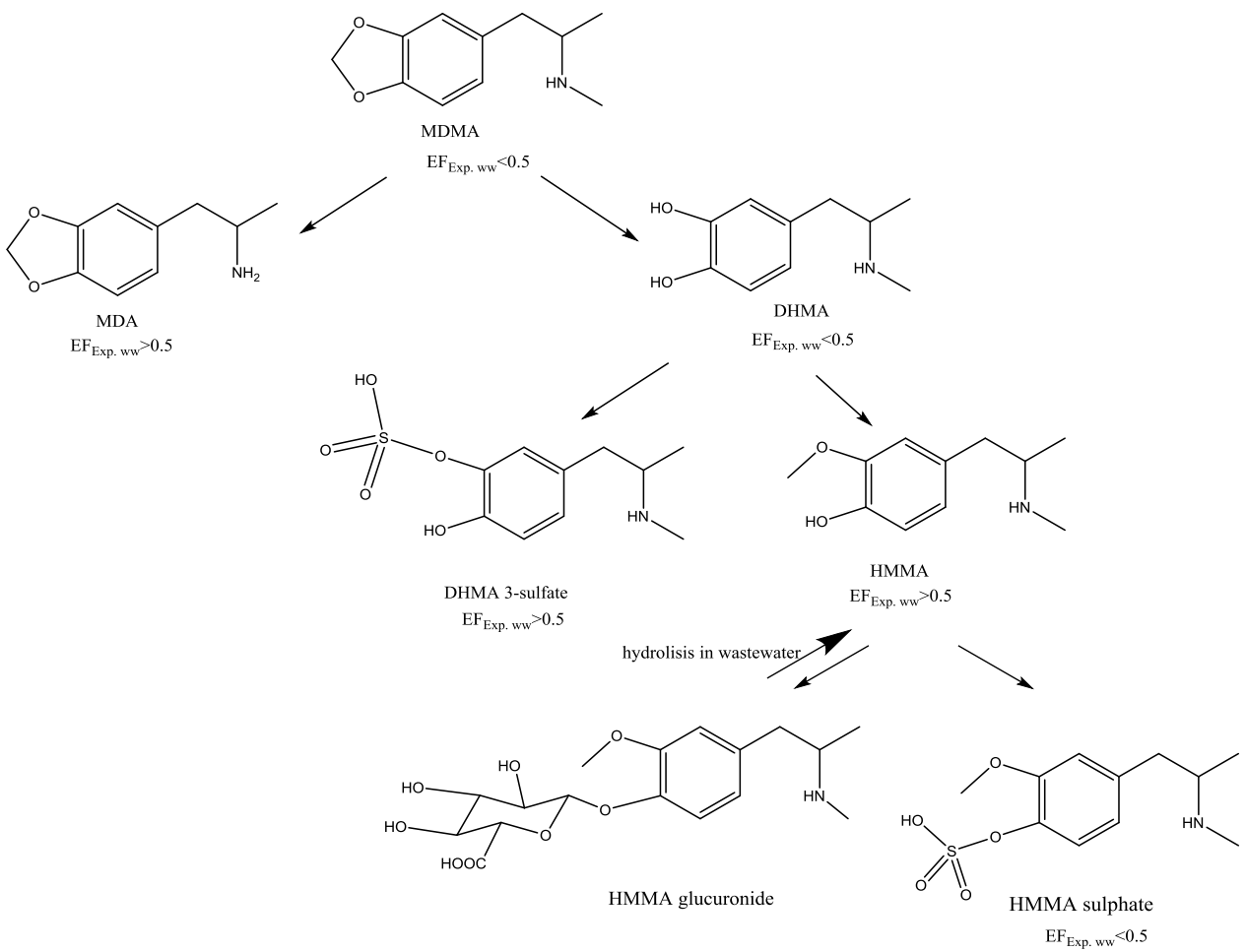



**Figure 5-5** Expected EF values in wastewater for MDMA consumption using the analytical conditions described in Castrignanò et al. EF was calculated by dividing the concentration of (+)-enantiomer and the sum of the concentrations of both enantiomers. DHMA, DHMA sulphate, HMMA glucuronide and HMMA sulphate were never detected in wastewater. The hypothesis is that HMMA glucuronide is hydrolysed by bacteria, giving HMMA enriched of the S-enantiomer.

Synthesis of MDMA follows many illicit syntheses pathways, such as Leuckart method or reductive aminations reactions, using safrole, isosafrole, piperonal and 3,4-methylenedioxyphenyl-2-propanone as starting reagents (these precursors are all listed). These methods produce racemic MDMA [49] and, therefore, they are not stereoselective [50].

This means that MDMA is illegally distributed as racemate. Consequently, if directly disposed of, it will be quantified as racemate in wastewater. Furthermore, MDMA is stereoselectively metabolised with preferential metabolism of *S*-(+)-MDMA, which is also eliminated faster than *R*-(-)-MDMA [51], and formation of MDA enriched with *S*-(+)-enantiomer [50]. Hence, if found in wastewater after consumption, MDMA will be enriched with *R*-(-)-enantiomer.

In the current study, population-normalised MDMA loads ranged from a minimum average value of 3.2 mg $day^{-1}$ 1000 $people^{-1}$ in Castellon to a maximum value of 62.0 mg $day^{-1}$ 1000 $people^{-1}$ in Utrecht. Increasing MDMA loads were found during weekend in all of the countries involved, with the exception of the Dutch city that had also high MDMA load on a weekday. The weekly average values were as follows:

- 37.7 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo. This value is half of 76.1 mg $day^{-1}$ 1000 $people^{-1}$ reported in 2014, but still higher of the loads in 2012 and 2013 (10.6 and 7.4 mg $day^{-1}$ 1000 $people^{-1}$ respectively) [17]. *R*-(-)-enantiomer was the predominant in wastewater samples (EF<0.5).
- 53.1 mg $day^{-1}$ 1000 $people^{-1}$ in Bristol, almost doubled respect to 31.4 mg $day^{-1}$ 1000 $people^{-1}$ reported in 2014 [17]. Wastewater samples were enriched with *R*-(-)-enantiomer (EF<0.5).
- 32.0 mg $day^{-1}$ 1000 $people^{-1}$ in Lyngby. This data is in agreement with the amount found in 2014 in Copenhagen (28.2 mg $day^{-1}$ 1000 $people^{-1}$ [17]). EF values were always below 0.5.
- 62.0 mg $day^{-1}$ 1000 $people^{-1}$ in Utrecht. This amount was lower respect to those ones found in the last two years in Utrecht (83.1 and 86 mg $day^{-1}$ 1000 $people^{-1}$ in 2013 and in 2014) and in 2012 (161.9 mg $day^{-1}$ 1000 $people^{-1}$) [17]. EF values were below 0.5.
- 21.9 mg $day^{-1}$ 1000 $people^{-1}$ in Brussels (15.3 and 13.0 mg $day^{-1}$ 1000 $people^{-1}$ were reported in 2013 and 2012 [17]). EF values always lower than 0.5.

- 43.5 mg day$^{-1}$ 1000 people$^{-1}$ in Zurich (42.9 and 55.4 mg day$^{-1}$ 1000 people$^{-1}$ were reported in 2013 and in 2014 [17]). The average EF was below 0.5 throughout all the week.
- 6.8 mg day$^{-1}$ 1000 people$^{-1}$ in Milan (less amounts were detected in 2013-14 [17]). The average EF was above 0.5 (high error bars were displayed).
- 3.2 mg day$^{-1}$ 1000 people$^{-1}$ in Castellon. Values were below LOQ in 2012, 6.4 mg day$^{-1}$ 1000 people$^{-1}$ in 2013 and 4.1 mg day$^{-1}$ 1000 people$^{-1}$ in 2014 [17]. EF values were < 0.5.

The overall MDMA weekly mean in 2013 was between 25 and 18 mg day$^{-1}$ 1000 people$^{-1}$ [5] (the first value corresponds to cities participating in all 3 years study, whilst the second one to all cities participating in 2013). A geographical trend of MDMA loads from North to South was also found. Indeed, Northern European cities (except for Brussels) were mostly above the average. Enantiomeric profiling revealed that MDMA in wastewater is enriched with *R*-(-)-MDMA (EF: 0.32 - 0.40). This indicates that MDMA retrieved in wastewater comes from consumption, due to stereoselective metabolism of MDMA in humans. Figure 5-5 shows expected EF values in wastewater for MDMA consumption using the analytical conditions described in Castrignanò et al [24]. Although illicit MDMA production sites are presumably mainly in the Netherlands and in Belgium (as mentioned in the EMCDDA report [28]), MDMA loads in these countries seem to be linked to a human consumption rather than its direct disposal. This is in contrast to a previous study in Utrecht, were high loads of racemic MDMA were recorded as indication of disposal of unconsumed drug [22]. The estimates of MDMA usage corrected by the CF ranged from the average of 93.0 mg day$^{-1}$ 1000 people$^{-1}$ in Oslo to 3.5 mg day$^{-1}$ 1000 people$^{-1}$ in Castellon.

The hypothesis that MDMA was present in European wastewaters as a result of its consumption was further evidenced by the study of MDA and its chiral signature. MDA can be a drug of abuse itself or a metabolite of MDMA and MDEA (3,4-methylenedioxyethylamphetamine). It is therefore of utmost importance to verify the origin of MDA. MDA does not have any medical applications and is available on the illicit marked as racemate [52]. This is due to non-stereoselective synthetic route. Similarly to MDMA, MDA's metabolism favours *S*-(+)-enantiomer [53]. Therefore, if MDA is consumed, it will be excreted in urine enriched with *R*-(-)-enantiomer. On the other hand, if MDA is formed as a result of the metabolism

of MDMA or MDEA, it will be present in urine (and in wastewater) enriched with *S*-(+)-enantiomer [54, 55]. In this study, MDEA was not detected in any European locations. The highest loads of MDA were recorded in Utrecht with 3.2 mg $day^{-1}$ 1000 $people^{-1}$, followed by Bristol with 1.9 mg $day^{-1}$ 1000 $people^{-1}$ and in Oslo with 0.5 mg $day^{-1}$ 1000 $people^{-1}$ at average weekly loads (Table S11). Interestingly, these countries have also high MDMA use, which led us to the conclusion that MDA could be present in wastewater due to consumption of MDMA. MDA was detected one day only in Brussels and Lyngby, respectively enriched of the *S*-(+)-form and *R*-(-)-form. Furthermore, enantioselective analysis revealed that MDA quantified in wastewater was, in most cases, enriched with *S*-(+)-enantiomer. This indicates that its presence is associated with the consumption of MDMA rather than the abuse of MDA. However, on two occasions, MDA was found enriched with *R*-(-)-enantiomer. This could indeed indicate an abuse of MDA. In the case of racemic MDA, this could indicate a combination of either the consumption of MDA and MDMA or simply the direct disposal of non-consumed MDA.

As MDA is a minor and not exclusive metabolite of MDMA, other metabolites were also considered as possible DTRs for MDMA consumption: HMA and HMMA.

HMA was detected at 3.4 mg $day^{-1}$ 1000 $people^{-1}$ as weekly average in three days of the monitoring week in the Dutch city (Saturday, Sunday and Monday) and at 7.4 mg $day^{-1}$ 1000 $people^{-1}$ in two days in Bristolian samples (Sunday and Monday) (Table S12). Because of the percentage of excretion of HMA after a dose of MDMA is 1.3%, its choice as MDMA DTR could be considered only in the case of high amount of MDMA intake. Indeed, it was found only in those places reporting the highest levels of MDMA. EF showed values close to 0.5 when high HMA loads were detected. However, the relevance of enantiomeric significance is difficult to comment because of the low number of positive samples.

HMMA, on the other hand, was found in wastewater at nanogram per litre level in six cities out of eight studied (i.e. no HMMA was detected in Oslo and Milan) (Table S13). HMMA's percentage excretion is 40%, which indicates that this metabolite could be used as MDMA's DTR. Due to stereoselective metabolism of MDMA favouring *S*-(+)-enantiomer, HMMA and its glucuronide derivative are formed enriched with *S*-(+)-enantiomer. Interestingly, HMMA sulphate is formed via non-stereoselective route [56]. In this study, HMMA was enriched of the second

eluting enantiomer. Assuming the same elution order of MDMA enantiomers for HMA and HMMA under the same chromatographic conditions, the second-eluting enantiomer could be assigned as *S*-(+)-enantiomer. The expected EF of HMMA in wastewater would be higher than 0.5. Therefore, we hypothesize that if an enrichment of *R*-(-)-MDMA occurred in case of consumption, the presence of *S*-(+)-HMMA would be observable along with an EF>0.5. Indeed, the trend observed for HMMA loads was quite superimposable to the parent drug MDMA, except for Oslo.

### 5.4.3 Mephedrone

Mephedrone is a stimulant semisynthetic derivative of cathinone. It was first synthesised in 1929 by Saem de Burnaga Sanchez but its abuse was documented for the first time only in 2007 [57]. Abuse of mephedrone was reported in several European countries. Recently, several mephedrone abuse associated deaths were reported in the UK [58]. In response to this, several modified cathinones were included in the UK Misuse Drugs Act (in category class B) in April 2010. Four fatalities due to mephedrone intake were confirmed in Scotland between February and May 2010 [59].

Mephedrone is a chiral compound. It contains one chiral carbon and, thus, it exists in two enantiomeric forms as *R*-(+)-mephedrone and *S*-(-)-mephedrone. Both enantiomers are characterised by different potency: *R*-(+)-mephedrone shows predominant dopaminergic action and stimulant-like properties than *S*-(-)-mephedrone [60]. Mephedrone can be synthesised via both non-stereoselective and stereoselective methods, but, as reported by EMCDDA [61] and confirmed in our previous chapter [24], the 'street mephedrone' is distributed as racemate. Metabolism of mephedrone is stereoselective favouring *R*-(+)-enantiomer and leading to the enrichment of excreted mephedrone with *S*-(-)-enantiomer.

Mephedrone was reported by EMCDDA (EU Early Warning System) to have increased usage in the UK in 2014 [28]. Indeed, mephedrone was detected only in the UK. Because it has no medical use in Europe [61], its presence in wastewater can be attributed only to illegal disposal or consumption. Population-normalised mass loads ranged throughout a sampling week from 14.9 to 47.7 mg day$^{-1}$ 1000 people$^{-1}$ (Table S14). Increasing loads were found in weekend days rather than weekdays with a mean value of 25.6 ± 12.0 mg day$^{-1}$ 1000 people$^{-1}$. A similar trend

was observed in Castrignanò et al. [24]. Furthermore, as mephedrone quantified in wastewater was found to be enriched with R-(+)-enantiomer, this indicates that mephedrone was consumed rather than directly disposed of.

### 5.4.4 Other Drugs

The analyses of precursors, such as norephedrine and ephedrines, were performed for Oslo, Bristol, Utrecht (only norephedrine) and Milan. An alternative metabolic pathway of the amphetamine produces norephedrine. In neutral condition of pH, when amphetamine is found unchanged in urine at 30%, norephedrine is present at 2% [19]. Hence, its content could vary in acid and alkaline urine conditions. Norephedrine is also used as decongestant. *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine are a bronchodilator and a decongestant respectively, whilst their enantiomers have no medical use. According to the EMCDDA, the production based on ephedrine and pseudoephedrine is occurring in Central European countries, such as Czech Republic, Slovakia and Germany [18]. Unfortunately, no Central European cities were included in the study. Population-normalised norephedrine loads were 51 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo, 7.1 mg $day^{-1}$ 1000 $people^{-1}$ in Milan and 3.4 mg $day^{-1}$ 1000 $people^{-1}$ in Bristol (Table S15). In Utrecht, norephedrine was not detected. EF were 0.48, 1 and 0.56 respectively. Population-normalised *1R,2S*-(-)-ephedrine loads were 0.7 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo, 3.4 mg $day^{-1}$ 1000 $people^{-1}$ in Milan and 0.6 mg $day^{-1}$ 1000 $people^{-1}$ in Bristol (Table S16). Population-normalised *1S,2S*-(+)-pseudoephedrine loads were 21.2 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo, 35.7 mg $day^{-1}$ 1000 $people^{-1}$ in Milan and 96.4 mg $day^{-1}$ 1000 $people^{-1}$ in Bristol (Table S16). EF were 0.48, 1 and 0.56 respectively.

PMA, a phenylisopropylamine with hallucinogenic properties, was not found in any cities.

Drugs, such as the narcotic-like pain reliever tramadol and the antidepressant venlafaxine, were included as they can be potentially abused. Results for venlafaxine (VEN) and its metabolite desmethylvenlafaxine (DMV) are displayed in Table S17-S18. VEN has an excretion percentage of 5%, while DMV 29-48% [29]. The weekly average values were the following:

- 58.7 and 98.5 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Oslo. There were two weekdays in which DMV was higher in loads respect to the other days. EF values were slightly below 0.5 for VEN, while they were also >0.5 for DMV.
- 41.4 and 82.3 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Bristol. The trend was quite constant for both compounds. EF values were just above 0.5 for VEN, while there was a predominance of the first eluting DMV enantiomer (EF equals to 0.65).
- 99.6 and 221.6 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Lyngby. The trend was rather constant along the week and the enantiomeric composition was racemic for both compounds.
- 54.7 and 93.0 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Utrecht with pretty stable loads and EF=0.5.
- 206.3 and 297.7 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Brussels with some high peaks during the week. EF values were near to 0.5 for VEN, whilst 0.58 indicating a predominance of the first eluting DMV enantiomer.
- 108.0 and 185.4 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Zurich with steady loads and EF=0.5 for both compounds.
- 27.9 and 60.3 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Milan with alternant loads for a couple of days and EF approximately equals to 0.5 even if EF varied throughout the weekfor both compounds.
- 90.3 and 235.9 mg day$^{-1}$ 1000 people$^{-1}$ for VEN and DMV in Castellon with small variation in loads for VEN and EF=0.53 for VEN and EF=0.51 for DMV.

According to these results, the ratio parent:metabolite was 1:2 for the majority of countries, with the exception of Brussels and Castellon. The interpretation of the enantiomeric composition was not univocal because of the different EF values obtained.

The population-normalised tramadol loads were rather stable for all the considered cities, with the exception of the Belgian city in which a doubled-average peak was detected on Saturday (Table S19). High mass loads were found in Bristol and Lyngby, whilst the highest value was in Brussels (686.9 mg day$^{-1}$ 1000 people$^{-1}$). EF values ranged from 0.53 to 0.61, showing a predominance of the first eluting diastomer.

Even though high usage of zopiclone, fluoxetine and norfluoxetine were suspected, these drugs were not detected in some cases or <MQL in others. This is

probably due to the relatively high MDL values for zopiclone, fluoxetine and its metabolite norfluoxetine in the developed method.

## 5.5 Conclusions

This was the first time that the enantiomeric profiling of several chiral drugs was studied in different European cities during a one-week monitoring campaign in 2015. A new occurring drug of abuse mephedrone was reported in the UK with population-normalised mass loads up to 47.7 mg $day^{-1}$ 1000 $people^{-1}$. Moreover, the enrichment of *R*-(+)-enantiomer in wastewater suggested a stereoselective metabolism in humans or stereoselective microbial metabolic processes occurring in the environment. Further investigations are needed for looking at additional mephedrone biomarkers for WBE approach. A spatial difference in loads was observed between Northern and Southern European cities for amphetamine. EF values showed a slight enrichment of *R*-(-)-amphetamine in wastewater with the exception for some days in Zurich. Still controversial remains the interpretation of enantiomeric composition of amphetamine in wastewater. High methamphetamine mass loads were found in Norway, where also high seizures were seen according to the EMCDDA. *S*-(+)-methamphetamine was the predominant enantiomer found in wastewater samples probably because its stereoselective synthesis was preferred in the illicit manufacturing market in Central Europe. The unique exception was represented by Norwegian data that suggested a different illegal synthetic route (e.g. racemic methamphetamine produced in Lithuania and exported to Baltic countries). The prevalence of *R*-(-)-MDMA found in the wastewater revealed that MDMA was due to its direct consumption, even in those cities where illicit manufacturing sites are present. *S*-(+)-MDA originated from MDMA metabolism (especially during weekends) rather than MDA itself. As MDA is a minor MDMA metabolite, other metabolites were considered for looking at other possible MDMA DTRs, such as HMA and HMMA. The latter compound seemed to be a suitable MDMA DTR. A slight preponderance of the *S*-(+)-HMMA could represent MDMA abuse. The investigation of precursors showed that their presence was reasonably ascribed at their medical use. The antidepressant venlafaxine gave a ratio "parent compound:metabolite" of 1:2 for the majority of the countries. Controversial is the interpretation of the enantiomeric composition of both compounds due to different EF values. Very high tramadol loads were found in Brussels. Chiral analysis

showed an EF range between 0.53 and 0.61, indicating the predominance of the first eluting diastomer.

## 5.6 Contributions

Erika Castrignanò and Barbara Kasprzyk-Hordern planned and designed the study. Richard Bade, Lubertus Bijlsma, J. A. Baz-Lomba, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Adrian Covaci, Emma Gracia-Lor, Barbara Kasprzyk-Hordern, Juliet Kinyua, Ann-Kathrin McCall, Alexander L. N. van Nuijs, Christoph Ort, Benedek G Plósz, Pedram Ramin, Nikolaos I Rousis, Yeonsuk Ryu, Kevin V Thomas, Pim de Voogt, Ettore Zuccato and Felix Hernandez organised the collection of the wastewater samples from their local wastewater treatment plant. Erika Castrignanò analysed the samples and interpreted the results with contribution from Barbara Kasprzyk-Hordern.

## 5.7 Supplementary Data

The following supplementary data are contained in Appendix 3:

Table S1 Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, a extracted from (Moffat, Osselton et al. 2004), b predicted using ACD/labs software (http://www.chemspider.com).

Table S2 MRM transitions selected for studied analytes.

Table S3 Validation parameters - retention time, relative retention time, linearity range, correlation coefficient obtained from calibration curve and instrumental and method limits of detection and instrumental and method limits of quantification.

Table S4 Validation parameters - method precision.

Table S5 Validation parameters -instrumental precision.

Table S6 Validation parameters –ion suppression.

Table S7 SPE recovery for the studied analytes.

Table S8 Amphetamine loads.

Table S9 Methamphetamine loads.

Table S10 MDMA loads.

Table S11 MDA loads.

Table S12 HMA loads.

Table S13 HMMA loads.

Table S14 Mephedrone loads.

Table S15 Norephedrine loads.

Table S16 Ephedrines loads.

Table S17 Venlafaxine loads.

Table S18 Desmethylvenlafaxine loads.

Table S19 Tramadol loads.

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# Chapter 6: Multi-residue stereoisomeric analysis of human and veterinary chiral drugs in wastewater using chiral liquid chromatography coupled with tandem mass spectrometry

## 6.1 Summary

Quinolones are broad-spectrum antibacterials with clinical and veterinary applications. Their wide use has rapidly brought to an increasing antibiotic resistance developed by bacteria. The research on the potential biomarkers for quinolones consumption in wastewater-based epidemiology (WBE) could be a useful means to understand their inappropriate use in the monitored areas. Moreover, there is currently a lack of studies on quinolones enantiomeric profiling in the environment, which is important to investigate for understanding the role of each enantiomer in the environment and, in some cases, the correlation with their human stereoselective metabolism.

This chapter proposes a novel multi-residue stereoisomeric analytical method for the simultaneous analysis of 16 human and veterinary quinolones drugs (including an antifungal drug). For the first time, the investigation of (±)-ofloxacin with its main metabolites (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin, (±)-moxifloxacin, the precursor (±)-prulifloxacin with its active compound (±)-ulifloxacin, (±)-*cis*-ketoconazole, (±)-flumequine, (±)-nadifloxacin and *R*-(+)-besifloxacin was carried out at enantiomeric level in composite wastewater samples along with their chiral separation. Chiral liquid chromatography coupled with tandem mass spectrometry was used as a tool of investigation. In particular, excellent chiral separation was achieved by using a CHIRALCEL® OZ-RH column. Enantiomeric resolution values higher than one ($R_s \geq 1$) were reached for six over nine chiral drugs enabling quantitative analysis. The overall performance of the method was satisfactory for all the targeted analytes with a method precision <20% and relative recoveries between 70% and 120%. Method detection limits were at nanogram per litre level. The method was applied to 24-hours composite samples from a week monitoring campaign of a large wastewater treatment plant in the South West of the UK. Many target pharmaceuticals were found at quantifiable concentrations in the analysed samples. Enantiomeric profiling revealed that (±)-ofloxacin was found enriched with *S*-(-)-enantiomer, due to its higher enantiomerically pure usage with respect to its enantiomer. Its urinary metabolites were found below the method quantification limit. (±)-*cis*-Ketoconazole was enriched with the first-eluting enantiomer.

## 6.2 Introduction

Quinolones are a family of synthetic broad-spectrum antibiotic drugs used for clinical and veterinary treatments. They belong to the class of inhibitors of bacterial topoisomerases IV [1] and DNA gyrase, the originally recognised drug target [2]. They are largely used for infections of urinary tract, bone and soft tissue, gastrointestinal tract, respiratory tract and sexually transmitted diseases [3]. Earliest members of this group were originally potent against Gram-negative bacteria, whilst new generations of fluorinated quinolones enlarged their potency range against Gram-positive and anaerobic bacteria [4]. Their wide use has rapidly led to an increasing antibiotic resistance developed by bacteria [5]. So far, three mechanisms have been described for explaining quinolones resistance: (i) target

alterations; (ii) decreased accumulations and (iii) mobile quinolones resistance element, such as plasmids, carrying qnr gene (less extent) [5]. Rate of resistance may vary by organisms and geographic areas [6]. In order to avoid the spread of resistance, a systematic surveillance program on antibiotic consumption and antimicrobial resistance (AMR) is essential. At the same time, early monitoring is desirable with the aim of getting real-time data. A possible way that enables real time estimation of antibiotics consumption can be represented by wastewater-based epidemiology (WBE). This approach relies on the detection and quantification of indicators, so-called biomarkers, which give a profile in terms of real usage of a substance through unique human urinary excretion pattern. Examples of this approach are well documented in the spatial and temporal community-wide monitoring of drugs of abuse [7, 8], alcohol [9] or tobacco use [10]. Indeed, the research on potential biomarkers of quinolones use suitable for WBE application can help in finding out their inappropriate use in the monitored areas and, thus, in informing the public health. The selection process of potential biomarkers is based on a full understanding of human pharmacokinetics along with stability properties in the environmental matrix. In addition, as some of these drugs are often administered as racemate, stereoselective metabolism and/or stereoselective enrichment or depletion of the enantiomeric composition of the drug and/or transformation products can occur respectively in humans and in the environment. In general, pharmacokinetic parameters, along with antibacterial activity, are influenced by the position of the chiral centre with respect to the quinolone ring. One of the exceptions is represented by (±)-lomefloxacin, in which the enantiomers do not show particularly differences in pharmacokinetics and activity [11]. Furthermore, chirality is an important aspect to investigate as it allows: (i) differentiating between the use and the misuse, (ii) distinguishing the origin of a drug residue through the distinction between consumption and disposal of unused drugs.

Additionally, enantiomeric profiling of quinolones in the environment has never been undertaken before. In order to undertake enantiomeric profiling of wastewater for chiral drug biomarkers, robust and multi-residue chiral analytical methods need to be developed [12]. Chiral LC-MS (liquid chromatography coupled with tandem mass spectrometry) methods are preferred methods as they allow quantitative as well as sensitive and selective measurements of the chiral drugs at

enantiomeric level [13]. Chiral LC-MS/MS methods have the advantage to combine the resolving power of HPLC and the features of sensitivity and specificity of MS [14]. Furthermore, the choice to use reversed-phase LC with MS detection allows: (i) handling the sample easily; (ii) choosing the proper buffer for controlling the selectivity and (iii) selecting compatible MS mobile phases [14]. Currently, multi-residue chiral LC-MS methods in environmental matrices are limited [12, 15-18] and, to the authors' knowledge, no reports are available in literature on the analysis and the enantiomeric profiling of quinolones and fluoroquinolones.

This chapter proposes a novel multi-residue stereoselective method for the simultaneous analysis of 16 human and veterinary quinolones drugs (including an antifungal drug). For the first time the investigation of (±)-ofloxacin with its main metabolites (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin, (±)-moxifloxacin, the prodrug (±)-prulifloxacin with its active compound (±)-ulifloxacin, (±)-cis-ketoconazole, (±)-flumequine, (±)-nadifloxacin and *R*-(+)-besifloxacin was carried out at enantiomeric level in composite wastewater samples along with their chiral separation. Moreover, the following achiral quinolones were included for monitoring purposes: ciprofloxacin, desethylene-ciprofloxacin, norfloxacin and nalidixic acid. Moxifloxacin-*N*-sulphate (with a defined stereochemistry) was also included as part of the urinary excretion profile of *S,S*-moxifloxacin.

## 6.3 Experimental

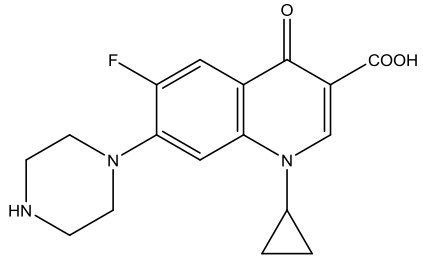
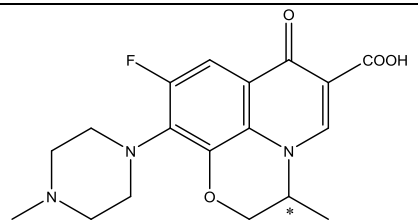
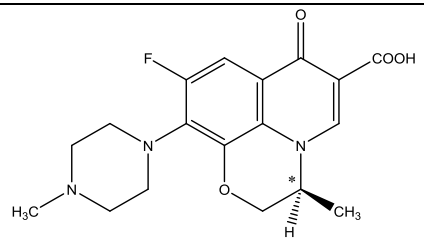
### 6.3.1 Chemicals and Materials

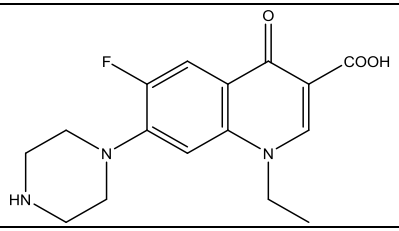
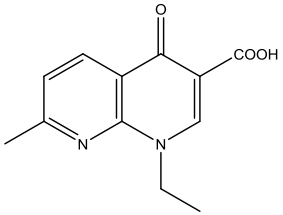
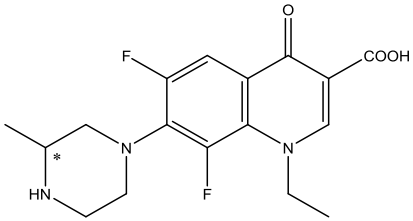
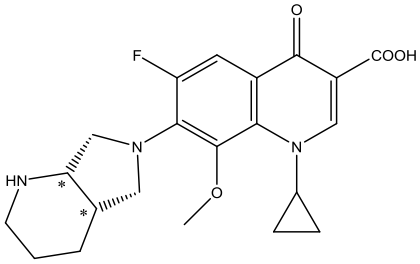
Table 6-1 shows the selection of the analytes considered in this study with information on their chemical structure, chirality, marketing, use, metabolic and excretion patterns, stereoselective metabolism.

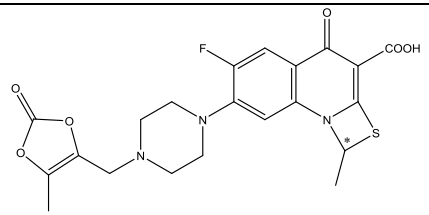
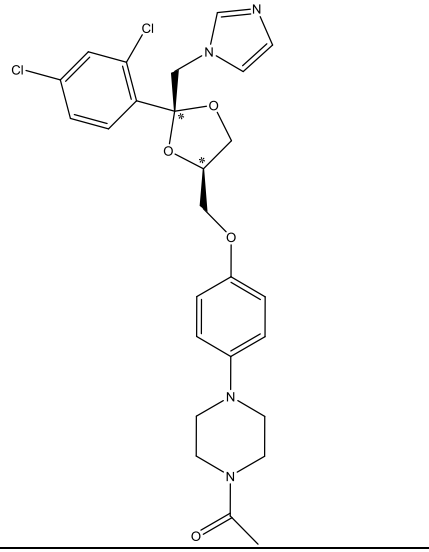
Table S1 shows CAS number, molecular formula, molecular weight, log P, $pK_a$ values and supplier information for all targeted analytes.

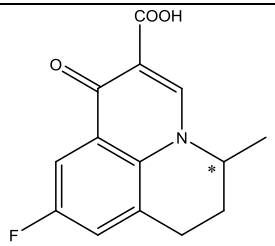
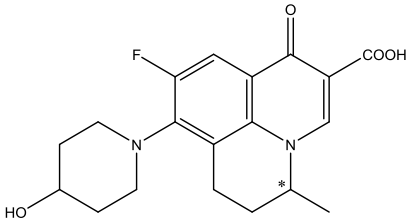
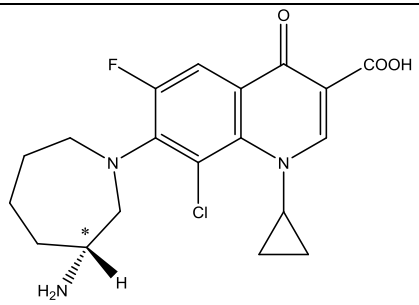
High pure grade standard solutions of achiral analytes were as follows: ciprofloxacin, desethylene-ciprofloxacin, norfloxacin and nalidixic acid. The following analytes were used as racemates: (±)-ofloxacin, (±)-ofloxacin-*N*-oxide, (±)-desmethyl-ofloxacin, (±)-lomefloxacin, (±)-prulifloxacin, (±)-ulifloxacin, (±)-ketoconazole, (±)-flumequine, (±)-nadifloxacin.

**Table 6-1** Selected chiral drug biomarkers (in italics) and their pharmacokinetic data

| Drug | Structure | Chirality | Marketing | Use | Metabolite | Excretion | Reference |
|---|---|---|---|---|---|---|---|
| *Ciprofloxacin* |  | No | Synthetic | Human | Parent compound<br>*Desethylene-ciprofloxacin*<br>Sulfo-ciprofloxacin<br>Oxo-ciprofloxacin | 40-50% | [19], [20] |
| *(±)-Ofloxacin* |  | Yes, 1*C | Synthetic | Human | Parent compound<br><br>*Desmethyl-ofloxacin*<br><br>*Ofloxacin-N-oxide* | In urine over 24-48 h and between 4-8% excreted in faeces<br>Small amount of the dose<br>Small amount of the dose | [21], [22] |
| | | | | | | Stereoselective metabolism | [23], [24], [25] |
| *S-(-)-Ofloxacin (L-Ofloxacin)* |  | Yes, 1*C | Synthetic | Human | Parent compound<br><br>*Desmethyl-levofloxacin*<br>*Levofloxacine-N-oxide* | In urine (80% to 85%) and in faeces (2%) within 24 h<br>2% of the dose<br>2% of the dose | [21] |
| *Norfloxacin* | | No | Synthetic | Human | Parent compound | 25-40% of the dose is excreted in urine, 30% (range: 10-50%) is excreted in feces within 48 hours | [26] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| |  | | | | Metabolites | 5-10% as metabolites within 24-48 hours | |
| *Nalidixic acid* |  | No | Synthetic | ? | Parent compound<br>7-hydroxynalidixic acid (active)<br>Glucuronide conjugates of 7-hydroxynalidixic acid (inactive)<br>Glucuronide conjugates of nalidixic acid (inactive)<br>7-carboxy metabolite (inactive) | 2-3% in the urine<br>About 80% of a dose is excreted in the urine in 8h, mainly as glucuronide conjugates | [21] |
| *(±)-Lomefloxacin* |  | Yes, 1*C | Synthetic | Human | Parent compound<br>Glucuronide | 65% in urine<br>9%<br><br>No info on stereoselective metabolism (to author's knowledge) | [27], [28] |
| *(±)-Moxifloxacin* |  | Yes, 2*C | Synthetic, sold in one form S,S-Moxifloxacin (shown on the left), its impurity is R,R[a] | Human | Parent compound<br><br>Moxifloxacin acyl glucuronide | ~20% in urine and ~25% in feces.<br><br>14% of the dose in urine | [19], [29], [30] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | *Moxifloxacin-N-sulphate* | 35% of the dose in faeces | |
| | | | | | | No info on stereoselective metabolism (to author's knowledge) | |
| *(±)-Prulifloxacin* |  | Yes, 1 *C | Synthetic prodrug, sold as racemate | Human | *Ulifloxacin*<br><br>Inactive metabolites | 17-23% in the urine and 17-29% in the faeces<br>7% | [31] |
| | | | | | | No stereoselective metabolism | [32] |
| *(±)-Ketoconazole* |  | Yes, 2 *C | Synthetic, sold as a racemate of the cis-configuration | Human/Veterinary | Parent compound | 2-4% in urine of 13% excreted in urine | [33], |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *(±)-Flumequine* |  | Yes, 1*C | Racemic | Veterinary [34] | Parent compound<br><br>7-hydroxy-flumequine<br><br>Glucuronides of flumequine | 81-86% in calves urine (after enzyme deconjugation)<br>12-17% in calves urine (after enzyme deconjugation)<br><br>Stereoselective metabolism in sheep, cattle and poultry | [35]<br><br>[36] |
| *(±)-Nadifloxacin* |  | Yes, 1*C | Synthetic | Human | Parent compound<br><br>Sulphates<br><br>Glucuronides | 0.09% of the administered dose was excreted in the urine over 48 hours, <5% eliminated in the urine, 20% as conjugates<br><br>No info on stereoselective metabolism (to author's knowledge) | [37], [38] |
| *R-(+)-Besifloxacin* |  | Yes, 1*C | Synthetic, sold in one form only | Human | Parent compound | 73% in animal feces, and 23% in animal urine.<br>No appreciable metabolism<br><br>No info on stereoselective metabolism (to author's knowledge) | [19], [39] |

[a] http://www.rxlist.com

Stereoisomerically pure standard solutions of the following analytes were used: *S*-(-)-ofloxacin, also known as levofloxacin, *R,R*-moxifloxacin, *S,S*-moxifloxacin and *S,S*-moxifloxacin-*N*-sulphate with two defined stereocentres and *R*-(+)-besifloxacin.

The following deuterated and isotopic analogues of target analytes were used as isotopically-labelled internal standards (ILIS): ciprofloxacin-$D_8$, (±)-ofloxacin-$D_3$, (±)-desmethyl-ofloxacin-$D_8$ and (±)-flumequine$^{13}C_3$.

Standard stock solutions were prepared at 1 mg $mL^{-1}$ concentration in methanol for all the analytes, except for (±)-prulifloxacin, (±)-ulifloxacin, (±)-ofloxacin-$D_3$ and (±)-flumequine$^{13}C_3$ that were dissolved in acetonitrile, (±)-lomefloxacin, desethylene-ciprofloxacin, ciprofloxacin-$D_8$ and (±)-desmethyl-ofloxacin-$D_8$ in water.

Working solutions of each stereoisomerically pure standard available were prepared at 50 ng $mL^{-1}$ in methanol and analysed to elucidate the elution order of (±)-ofloxacin and its metabolites enantiomers, and (±)-moxifloxacin diastereoisomers. Mixed working solutions containing all analytes were prepared from stock solutions at different concentration levels by dilution with mobile phase. They were used for the preparation of the aqueous standard calibration solutions and for spiking samples in the validation study. Stock and working solutions of standards were stored at -20° C.

HPLC-grade methanol (MeOH), acetonitrile (ACN), isopropanol (IPA), ammonium formate and formic acid (≥96%) were purchased from Sigma Aldrich, UK, ethanol (EtOH) from Fluka, UK. Ultrapure water was obtained from a MilliQ system, UK. All glassware was deactivated in order to prevent the adsorption of polar compounds to the hydroxyl sites on the glass surface. The deactivation process consisted of rinsing cycles with 5% DMDCS once, with toluene twice and with methanol thrice.

### 6.3.2 Sample collection, storage and preparation

24h time-proportional (10 mL every 15 minutes) composite wastewater influent samples were collected in PTFE bottles (Fisher, UK) in an autosampler ISCO 3700 from a wastewater treatment plant in the South West of the UK during one week-monitoring campaign in 2015. They were transported to the laboratory in

cool boxes packed with ice blocks and filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK). 50 µL of a mixture of ILIS at concentration of 1 mg $L^{-1}$ were added to 50 mL of a wastewater sample to provide final concentration of 1 µg $L^{-1}$. Analytes were extracted using SPE and Oasis HLB cartridges (60 mg, Waters, UK), previously conditioned with 3 mL of methanol and equilibrated with 3 mL of ultrapure water at a rate of 3 mL $min^{-1}$. 50 mL of spiked environmental samples were passed through the HLB cartridges at a rate of 8 mL $min^{-1}$. The cartridges were then washed with 1 mL of ultrapure water at a rate of 3 mL $min^{-1}$. The elution was carried out with 4 mL of methanol at a rate of 8 mL $min^{-1}$ into 5 mL silanised glass tubes. The extracts were transferred to the TurboVap evaporator (Caliper, UK) and completely evaporated to dryness under nitrogen flow (5-10 psi). Samples were reconstituted with 0.5 mL of 10 mM ammonium formate/methanol 1:99 v/v with 0.05% formic acid and filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm). The filtered samples were transferred to polypropylene plastic vials bonded pre-slit PTFE/Silicone septa (Waters, UK) and then 20 µL were directly injected into a chiral HPLC-MS/MS system. Samples from the monitoring campaign were prepared and analysed in duplicate.

### 6.3.3 Sample analysis by chiral liquid chromatography coupled with tandem mass spectrometry

Samples were analysed using a Waters ACQUITY UPLC® system (Waters, Manchester, UK).

Chromatographic separation of all the analytes was carried out using a chiral CHIRALCEL® OZ-RH column (5 µm particle size, L × I.D. 15 cm × 2.1 mm, Chiral Technologies, France) with a 2.0 mm × 2.0 mm guard filter (Chiral Technologies, France). The column temperature was set at 30°C. The autosampler was kept at 4°C.The injection volume of the sample was set at 20 µL.

Several mobile phase compositions differing in organic modifier, variable percentage of organic modifier used and mixture of organic modifiers were tested (Table S2). Different mobile phase flow rates (from 0.05 mL $min^{-1}$ to 0.2 mL $min^{-1}$) were also trialled. The best chiral recognition and chromatographic separation of analytes was achieved with a mobile phase composed of 10 mM ammonium formate/methanol 1:99 v/v with 0.05% formic acid at a flow rate of 0.1 mL $min^{-1}$ under isocratic conditions.

The MS system was a triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an electrospray ionisation source (ESI). Analyses were performed in positive mode with an optimised capillary voltage of 3 kV, source temperature of 350°C, desolvation temperature of 350°C and desolvation gas flow of 650 l $h^{-1}$. Nitrogen, supplied by a high purity nitrogen generator (Peak Scientific, UK), was used as a nebulising and desolvation gas. Argon (99.999%) was used as a collision gas. MassLynx 4.1 (Waters, UK) was used to control the Waters ACQUITY system and the Xevo TQD. Data processing was carried out on TargetLynx software (Waters, Manchester, UK).

The mass spectrometer acquired data using MRM mode, which enabled the measurement of the fragmentation of the protonated pseudo-molecular ions of each compound. The choice of fragmentation ion for each compound was based on the most intense signal. Corresponding CVs and CEs were obtained after direct infusion of each standard at a concentration of 100 µg $L^{-1}$ into the mass spectrometer. Their optimisation for the chosen MRM transitions was carried out using mobile phase facilitating the best analytical performance by infusing each standard at 100 µg $L^{-1}$ combined with LC conditions. Two MRM transitions were selected for each compound. The most abundant transition product ion was typically used for quantification, whilst the second ion was used for confirmation purposes. The MRM transitions, CV and CE values of the studied compounds are presented in Table 2.

Selection of ILIS (see Table 6-2) for those compounds for which deuterated or $^{13}C$ analogues were not available in our laboratory was based on similar chemical structure and elution time to account for possible signal suppression or enhancement of studied analytes in ESI. The most suitable ILIS was chosen for each substance.

### 6.3.4 Method validation

The method validation was performed in agreement with European Guidelines concerning the performance of analytical methods and the interpretation of results [40].

**Table 6-2** MRM transitions selected for studied analytes and internal standards.

| Compound | CV/CE[a] | MRM1 (quantification) | CV/CE[a] | MRM2 (confirmation) | MRM1/MRM2 ratio ± SD | Internal standard |
|---|---|---|---|---|---|---|
| Ciprofloxacin | 42/40 | 332.2 > 231.1 | 42/32 | 332.2 > 245.1 | 8.9 ± 2.2 | Ciprofloxacin –$D_8$ |
| Desethylene-ciprofloxacin | 40/34 | 306.3 > 217.1 | 40/26 | 306.3 > 268.0 | 1.4 ± 0.4 | Ciprofloxacin –$D_8$ |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 20/32 | 362.2 > 261.2 | 20/32 | 362.2 > 318.7 | 29.6 ± 3.4 | *S*-(-)-Ofloxacin-$D_3$ |
| *R*-(+)-Ofloxacin | 20/32 | 362.2 > 261.2 | 20/32 | 362.2 > 318.7 | 30.0 ± 3.0 | *R*-(+)-Ofloxacin-$D_3$ |
| Norfloxacin | 58/26 | 320.2 > 233.1 | 58/38 | 320.2 > 204.9 | 2.6 ± 0.5 | Ciprofloxacin –$D_8$ |
| *S*-(-)-Ofloxacin-N-oxide | 28/18 | 378.3 > 316.7 | 28/44 | 378.3 > 246.9 | 2.7 ± 0.2 | *S*-(-)-Ofloxacin-$D_3$ |
| *R*-(+)-Ofloxacin-N-oxide | 28/18 | 378.3 > 316.7 | 28/44 | 378.3 > 246.9 | 2.9 ± 0.4 | *R*-(+)-Ofloxacin-$D_3$ |
| *S*-(-)-Desmethyl-ofloxacin | 50/26 | 348.2 > 261.0 | 50/33 | 348.2 > 221.0 | 7.1 ± 0.6 | *S*-(-)-Desmethyl-ofloxacin-$D_8$ |
| *R*-(+)-Desmethyl-ofloxacin | 50/26 | 348.2 > 261.0 | 50/33 | 348.2 > 221.0 | 7.2 ± 0.7 | *R*-(+)-Desmethyl-ofloxacin-$D_8$ |
| Nalidixic acid | 30/28 | 233.2 > 187.0 | 30/28 | 233.2 > 215.1 | 5.6 ± 0.3 | Ciprofloxacin –$D_8$ |
| (±)-Lomefloxacin | 22/24 | 352.0 > 265.0 | 22/22 | 352.0 > 308.0 | 3.0 ± 0.2 | Ciprofloxacin –$D_8$ |
| *R,R*-Moxifloxacin | 54/27 | 402.2 > 364.0 | 54/23 | 402.2 > 261.0 | | *S*-(-)-Desmethyl-ofloxacin-$D_8$ |
| *S,S*- Moxifloxacin | 54/27 | 402.2 > 364.0 | 54/23 | 402.2 > 261.0 | | *R*-(+)-Desmethyl-ofloxacin-$D_8$ |
| Moxifloxacin-N-sulphate | 54/27 | 402.2 > 364.0 | 54/28 | 402.2 > 341.0 | 2.8 ± 0.8 | *S*-(-)-Desmethyl-ofloxacin-$D_8$ |
| Prulifloxacin-E1 | 42/22 | 462.2 > 444.1 | 42/32 | 462.2 > 360.1 | 1.2 ± 0.1 | *S*-(-)-Ofloxacin-$D_3$ |
| Prulifloxacin-E2 | 42/22 | 462.2 > 444.1 | 42/32 | 462.2 > 360.1 | 1.2 ± 0.2 | *R*-(+)-Ofloxacin-$D_3$ |
| Ulifloxacin-E1 | 42/22 | 350.2 > 306.4 | 42/26 | 350.2 > 263.0 | 1.2 ± 0.3 | *S*-(-)-Desmethyl-ofloxacin-$D_8$ |
| Ulifloxacin-E2 | 42/22 | 350.2 > 306.4 | 42/26 | 350.2 > 263.0 | 1.2 ± 0.2 | *R*-(+)-Desmethyl-ofloxacin-$D_8$ |
| (±)-*cis*-Ketoconazole-E1 | 60/50 | 532.0 > 82.0 | 60/58 | 532.0 > 112.1 | 3.3 ± 0.1 | *S*-(-)-Ofloxacin-$D_3$ |
| (±)-*cis*-Ketoconazole-E2 | 60/50 | 532.0 > 82.0 | 60/58 | 532.0 > 112.1 | 3.4 ± 0.1 | *R*-(+)-Ofloxacin-$D_3$ |
| Flumequine-E1 | 28/34 | 262.2 > 201.9 | 28/26 | 262.2 > 244.5 | 1.7 ± 0.1 | Flumequine-$^{13}C_3$-E1 |
| Flumequine-E2 | 28/34 | 262.2 > 201.9 | 28/26 | 262.2 > 244.5 | 1.8 ± 0.2 | Flumequine-$^{13}C_3$-E2 |
| Nadifloxacin-E1 | 40/38 | 361.3 > 282.9 | 40/44 | 361.3 > 256.8 | 1.6 ± 0.1 | Flumequine-$^{13}C_3$-E1 |
| Nadifloxacin-E2 | 40/38 | 361.3 > 282.9 | 40/44 | 361.3 > 256.8 | 1.6 ± 0.2 | Flumequine-$^{13}C_3$-E2 |
| *R*-(+)-Besifloxacin | 34/14 | 394.1 > 376.4 | 34/24 | 394.1 > 356.0 | 3.4 ± 0.4 | Ciprofloxacin –$D_8$ |

| Internal Standard | CV/CE[a] | MRM1 (quantification) |
|---|---|---|
| Ciprofloxacin –$D_8$ | 30/26 | 340.1 > 296.1 |
| *S*-(-)-Ofloxacin-$D_3$ | 47/28 | 365.2 > 261.0 |
| *R*-(+)-Ofloxacin-$D_3$ | 47/28 | 365.2 > 261.0 |
| *S*-(-)-Desmethyl-ofloxacin-$D_8$ | 64/32 | 356.6 > 265.1 |
| *R*-(+)-Desmethyl-ofloxacin-$D_8$ | 64/32 | 356.6 > 265.1 |
| Flumequine-$^{13}C_3$-E1 | 40/24 | 265.1 > 246.9 |
| Flumequine-$^{13}C_3$-E2 | 40/24 | 265.1 > 246.9 |

[a]CV, cone voltage (V); CE, collision energy (eV)

The developed method was fully validated for wastewater samples by studying instrumental and method limits of detection and quantification, linearity, precision and accuracy, matrix effect.

IDL and IQL values were determined using a S/N approach at the minimum concentration value that provided S/N≥ 3 and S/N ≥ 10 for IDL and IQL respectively. MDL was calculated using the equation (4) in chapter 3. In this case, CF, that was the SPE concentration factor, was equal to 100. MQL was calculated using the equation (5) in chapter 3.

The linearity of the method was studied from 0 to 2000 μg $L^{-1}$ through the analysis of individual calibrators injected in triplicate. They were prepared in mobile phase at several concentration levels: 2000, 1000, 800, 700, 600, 500, 400, 300, 200, 100, 50, 10, 5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005 and 0 μg $L^{-1}$. ILIS approach was used for quantification purposes and for evaluating method performance.

Precision was expressed as relative standard deviation (RSD) of replicate analysis ($n$=4) at three different concentrations on the same day (intra-RSD%). In particular, instrumental precision was evaluated using standard solutions spiked in mobile phase at 10, 100 and 1000 μg $L^{-1}$ for achiral or not stereoisomerically separated analytes, at 5, 50 and 500 μg $L^{-1}$ in the case of individual enantiomers. Method precision was assessed using standard solutions spiked in 50 mL of influent wastewater at 50, 500 and 5000 ng $L^{-1}$ for achiral or not enantiomerically separated analytes, at 25, 250 and 2500 ng $L^{-1}$ in the case of individual enantiomers. Spiked wastewater samples were then subject to SPE as discussed in 6.3.2.

Reproducibility (inter-day precision) of the method was determined by replicate measurements ($n$=3) of the same concentrations of analytes as in the case of intra-day precision on three different days in order to assess the inter-day instrumental precision and the inter-day method precision. Precision data were acceptable when the RSD% was less than 20% for all the concentrations investigated during the different days. Quality controls at three concentration levels (10, 100 and 1000 μg $L^{-1}$) were also prepared and injected on regular basis to maintain instrument's performance.

Method accuracy was expressed as a percentage of closeness agreement between the difference from the concentration of a spiked sample before the extraction (with the background peak subtracted) and the peak area of a standard.

Sample carryover was studied by injecting spiked samples at a concentration of 2000 µg $L^{-1}$ followed by three blanks and it was considered insignificant if the concentration of the analyte was below the LOQ.

Matrix effect (ME) was calculated using the following equation (9):

$$ME_{without\_IL\ IS}\ [\%] = \left( \frac{A_{SPIKED\_AFT\ \ ER\_SPE} - A_0}{A_{AP}} \right) * 100 \qquad (9)$$

where $A_{SPIKED\_AFTER\_SPE}$ was the analyte peak area in wastewater extract spiked after SPE extraction, $A_0$ was the analyte peak area in the unspiked wastewater extract, $A_{AP}$ was the analyte peak area of standard spiked in aqueous phase. In order to correct matrix enhancement or suppression in some cases, ME was also calculated considering the response of ILIS as shown in equation (10):

$$ME_{with\_ILIS}\ [\%] = \left( \frac{\frac{A_{SPIKED\_AFT\ \ ER\_SPE}}{A_{ILIS\_SPIKE\ \ D\_AFTER\_SP\ \ E}} - \frac{A_0}{A_{ILIS\_0}}}{\frac{A_{AP}}{A_{ILIS\_AP}}} \right) * 100 \qquad (10)$$

where $A_{ILIS\_SPIKED\_AFTER\_SPE}$ was the ILIS peak area in wastewater extract spiked after SPE extraction, $A_{ILIS\_0}$ was the ILIS peak area in the unspiked wastewater extract and $A_{ILIS\_AP}$ was the ILIS peak area in the standard spiked in aqueous phase.

Absolute recovery, expressed as a percentage of the ratio between the peak area of analyte spiked before SPE ($A_{SPIKED\_BEFORE\_SPE}$) and that one spiked after SPE ($A_{SPIKED\_AFTER\_SPE}$) (both subtracted of the peak area of the unspiked sample), was calculated according to the following equation (11):

$$Abs\_recove\ \ ry\ [\%] = \left( \frac{A_{SPIKED\_BEF\ \ ORE\_SPE} - A_0}{A_{SPIKED\_AFT\ \ ER\_SPE} - A_0} \right) * 100 \qquad (11)$$

where $A_{SPIKED\_BEFORE\_SPE}$ was the peak area in wastewater extract spiked after SPE extraction, $A_{ILIS\_0}$ was the ILIS peak area in the unspiked wastewater extract and $A_{ILIS\_AP}$ was the ILIS peak area in the standard spiked in aqueous phase.

Relative recovery was calculated by taking into account the ILIS contribution as follows in equation (12):

$$SPE\_relative\_recovery\,[\%] = \left( \frac{\frac{A_{SPIKED\_BEFORE\_SPE}}{A_{ILIS\_SPIKED\_BEFORE\_SPE}} - \frac{A_0}{A_{ILIS\_0}}}{\frac{A_{SPIKED\_AFTER\_SPE}}{A_{ILIS\_SPIKED\_AFTER\_SPE}} - \frac{A_0}{A_{ILIS\_0}}} \right) * 100 \qquad (12)$$

where $A_{ILIS\_SPIKED\_BEFORE\_SPE}$ was the ILIS peak area in wastewater extract spiked before SPE.

Along with the above-mentioned parameters, resolution of enantiomers and stereoisomeric fraction were determined as key parameters for a chiral method.

Resolution of enantiomers of chiral drugs ($R_s$) was calculated using equation (7) reported in chapter 3. $R_s$ =1 indicates 2% overlap which is deemed acceptable for quantification purposes.

Enantiomeric fraction (EF) was calculated using equation (8) from chapter 3.

In the case of (±)-moxifloxacin, DF was calculated as follows in equation (13):

$$DF_{Moxifloxac\ in} = \frac{(S,S) - Moxifloxac\ in}{[(S,S) - Moxifloxac\ in + (R,R) - Moxifloxac\ in]} \qquad (13)$$

EF (or DF) equals 1 or 0 in the case of enantiomerically (or diasteromerically) pure compound, and 0.5 in the case of a racemate.

According to [40], the identification of the analyte was based on the following criteria: (i) the presence of all selected MRM transitions and (ii) a maximum permitted tolerance for relative ion intensities of MRM transitions not changing more than ±20% for ions with relative intensities of >50%, ±25% for ions with relative intensities between 20% and 50%, 30% for ions with relative intensities between 10% and 20% and ±50% for ions with relative intensities less than 10%.

## 6.4 Experimental

### 6.4.1 Selection of potential biomarkers

There are twenty-eight quinolones currently included among antiinfectives for systemic use [41]. All chiral quinolones were initially considered for the study.

However, some quinolones are not marketed in Europe. There are for example:(±)-gemifloxacin, which is a synthetic broad-spectrum drug developed as racemate, but not approved in Europe (Korea only);

- (±)-gatifloxacin, which is a synthetic racemic drug removed from the European market and currently available only in the US and Canada as an ophthalmic solution;
- (±)-danofloxacin,(±)-orbifloxacin, (±)-ibafloxacin and (±)-pradofloxacin, which are for veterinary use only;
- (±)-balofloxacin, which is available only in Korea;
- (±)-pazufloxacin, (±)-tosufloxacin and (±)-sitafloxacin, which are marketed only in Japan;
- (±)-garenoxacin, which is available in Korea, Japan and China.

The final list of target quinolones included: ciprofloxacin, desethylene-ciprofloxacin, (±)-ofloxacin, norfloxacin, (±)-ofloxacin-*N*-oxide, (±)-desmethyl-ofloxacin, nalidixic acid, (±)-lomefloxacin, *S,S*-moxifloxacin, *R,R*-moxifloxacin, moxifloxacin-*N*-sulphate, (±)-prulifloxacin, (±)-ulifloxacin, (±)-flumequine, (±)-nadifloxacin and *R*-(+)-besifloxacin (Table 6-1). (±)-*cis*-Ketoconazole was the only chiral antifungal drug included.

Ciprofloxacin and its metabolite desethylene-ciprofloxacin were targeted as biomarkers of ciprofloxacin use. The choice for indicators of (±)-ofloxacin consumption was based on human urinary excretion pattern of (±)-ofloxacin. Therefore, (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin were included, despite their low excretion factor. Nalidixic acid itself was selected as a biomarker for its usage despite the fact that only 2-3% of nalidixic acid is excreted unchanged. About 80% of a dose is mainly excreted in the urine as glucuronide conjugates, including glucuronides of 7-hydroxynalidixic acid. However, glucuronides of nalidixic acid can be hydrolysed by bacteria present in the wastewater, thus increasing the levels of nalidixic acid present. In the case of (±)-moxifloxacin, both the parent compound and the moxifloxacin-*N*-sulphate were considered. Since this drug is sold as enantiomerically pure *S,S*-moxifloxacin (Avelox is the trade name in the UK), also its impurity *R,R*-stereoisomer was investigated in order to verify any variations in a chiral signature of this compound. To the author's knowledge, no investigation of the enantiomeric profiling in wastewater of (±)-prulifloxacin

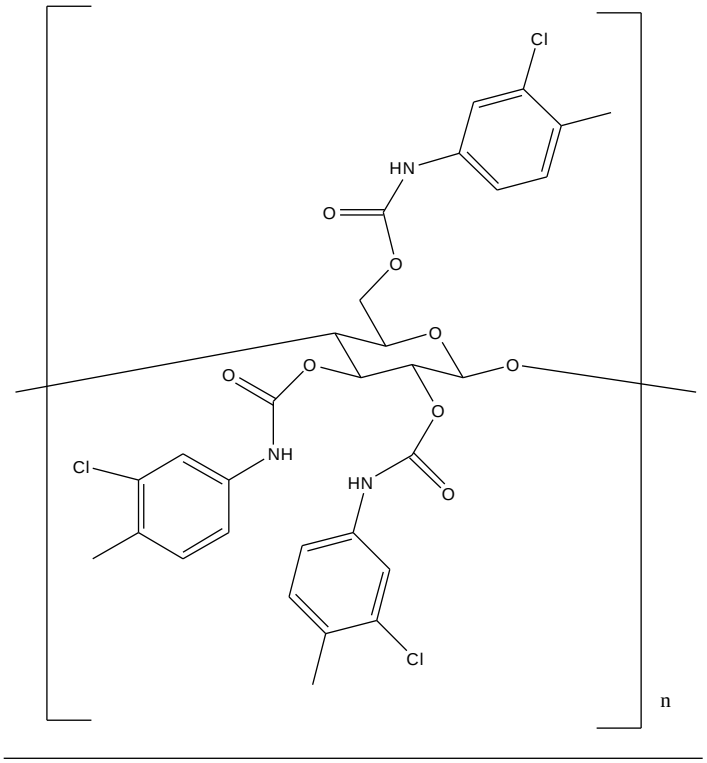
and (±)-ulifloxacin has been undertaken to date. (±)-Prulifloxacin is a synthetic prodrug sold as racemate for oral administration. It is converted in its active compound ulifloxacin by hepatic enzyme through the removal of the side chain in 4' site of piperazinyl, where the chiral centre is not the metabolic site. Only *L*-ulifloxacin has the bactericidal effects, but enantiomerically pure *L*-prulifloxacin is not commercially available yet [42]. The veterinary (±)-flumequine was included for verifying its presence in the environmental matrix. Racemic (±)-nadifloxacin is formulated in topical creams, although the antibacterial activity is higher for the isomer *S*-(-)-nadifloxacin. Even though <5% of nadifloxacin is excreted in urine, 20% is excreted as sulphate and glucuronides [38], which can be potentially re-converted to the parent compound in wastewater. *R*-(+)-besifloxacin is a synthetic quinolone sold only in one enantiomeric form that has ophtalmic use. It is metabolically stable and it does not go through chiral interconversion to its enantiomer after human hepatocytes incubation [39]. (±)-Ketoconazole (the only antifungal agent on the list) has two chiral centres and exists as four enantiomers [43]: *cis*-(2*S*,4*R*), *cis*-(2*R*,4*S*), *trans*-(2*R*,4*R*), *trans*-(2*S*,4*S*). Clinically, it is sold and administered as racemate of two *cis*-enantiomers, i.e. (+)-ketoconazole and (−)-ketoconazole [44].The parent compound itself was considered as biomarker of drug consumption for norfloxacin, (±)-lomefloxacin, (±)-nadifloxacin, *R*-(+)-besifloxacin and (±)-*cis*-ketoconazole.

### 6.4.2 Method development for the detection of quinolones and an antifungal drug in wastewater

#### 6.4.2.1 CHIRALCEL® OZ-RH column

The CHIRALCEL® OZ-RH column contains a cellulose tris (3-chloro-4-methylphenylcarbamate) as chiral selector coated on 5µm of silica gel. Its schematic structure is presented in Figure 6-1. This cellulose phenylcarbamate derivative belongs to the group of polysaccharide derivatives in the chiral stationary phases (CSPs) along with amylose phenylcarbamate derivatives [45]. Chiral recognition is mainly controlled by the presence of an electron-withdrawing substituent (in this case the halogen group) and an electron-donating substituent (the methyl group) in position 3 and 4 of the phenyl moiety, respectively [46, 47].

**Figure 6-1** CHIRALCEL® OZ-RH column – stationary phase modified from instruction manual provided by the supplier.




The nature of these substituents on the phenyl group of the polymer, along with their position [48, 49], produces an inductive effect on the carbamate group.

Consequently, the alteration of its polarity influences how cellulose derivatives interact with the chiral compound. In previous studies, it was demonstrated that the nature and the concentration of acidic additives were essential factors in determining the retention time and the enantioresolution of the analytes in polar organic solvent chromatography and normal phase liquid chromatography [47]. In the current study, reverse phase chromatography was used (see the advantages mentioned in paragraph 6.2).

The purpose of this method development was to investigate enantioseparation of the target compounds in order to obtain the best method's performance in terms of short time analysis and enantiomeric resolution within one analytical run.

The enantioselectivity of the retention was studied over a range of different organic modifiers, ratio organic:aqueous phase compositions, pH values, flow rates and percentages of additive.

The impact of organic content on the separation was evaluated in ACN, EtOH, MeOH, IPA and mixtures made of ACN:MeOH and EtOH:MeOH (Figure S1). A comparison of the impact of the mobile phases with the same percentage of organic on the retention time is shown in Figure S2a. The separation selectivity differed for protic and aprotic solvents, thus affecting the retention time of the analytes. Longer retention time was observed when the mobile composition consisted of an aprotic solvent such as ACN, followed by protic solvents such as IPA. The mixture EtOH:MeOH provided an adequate separation selectivity for ofloxacin and its metabolites only, but it was insufficient for the other compounds tested. The lowest retention times were observed for the mixture ACN:MeOH, but unsatisfactory enatioseparation was determined for the majority of the substances.

The impact of the nature of the organic content in the mobile phase on enantiomeric resolution was shown by considering the best performing mobile phases (95:5 5mM ammonium formate for IPA and ACN, 99:1 5mM ammonium formate for EtOH, 99:1 10mM ammonium formate for MeOH). Since enantiomeric resolution values preferably higher than one enable quantitative analysis at enantiomeric level, the aim of this method development phase was to obtain $R_s \geq 1$ for the majority of targeted compounds. A summary of the best performing mobile phases in terms of capability to enantioseparate the analytes is shown in Figure S2b. The study revealed that $R_s \geq 1$ was achieved for six compounds (ofloxacin, ofloxacin-*N*-oxide, prulifloxacin, flumequine, nadifloxacin and cis-ketoconazole) , $0.8 < R_s < 1$ in the case of two (desmethyl-ofloxacin and moxifloxacin) and $R_s > 0.6$ in one case (ulifloxacin) using 10 mM ammonium formate/MeOH 1:99 as mobile phase. $R_s \geq 1$ values were found for three analytes (ofloxacin, flumequine and nadifloxacin) and only one (flumequine) when EtOH and IPA were respectively used. For mobile phase containing ACN, $R_s > 0.6$ was obtained in one case only (flumequine). Moreover, higher enantioresolution values were observed for protic solvent mobile phase composition and they increased with the polarity of the protic solvent. Indeed, the highest Rs values were seen with MeOH-based mobile phase with respect to the less polar EtOH- and IPA-based. Furthermore, the enantioselectivity was

influenced by the water content in mobile phases. In fact, lower water content provided higher resolution of enantiomers. The highest Rs values were determined for nadifloxacin ($R_s$ = 2.86), followed by flumequine ($R_s$ = 1.91) and ofloxacin-N-oxide ($R_s$ = 1.71).

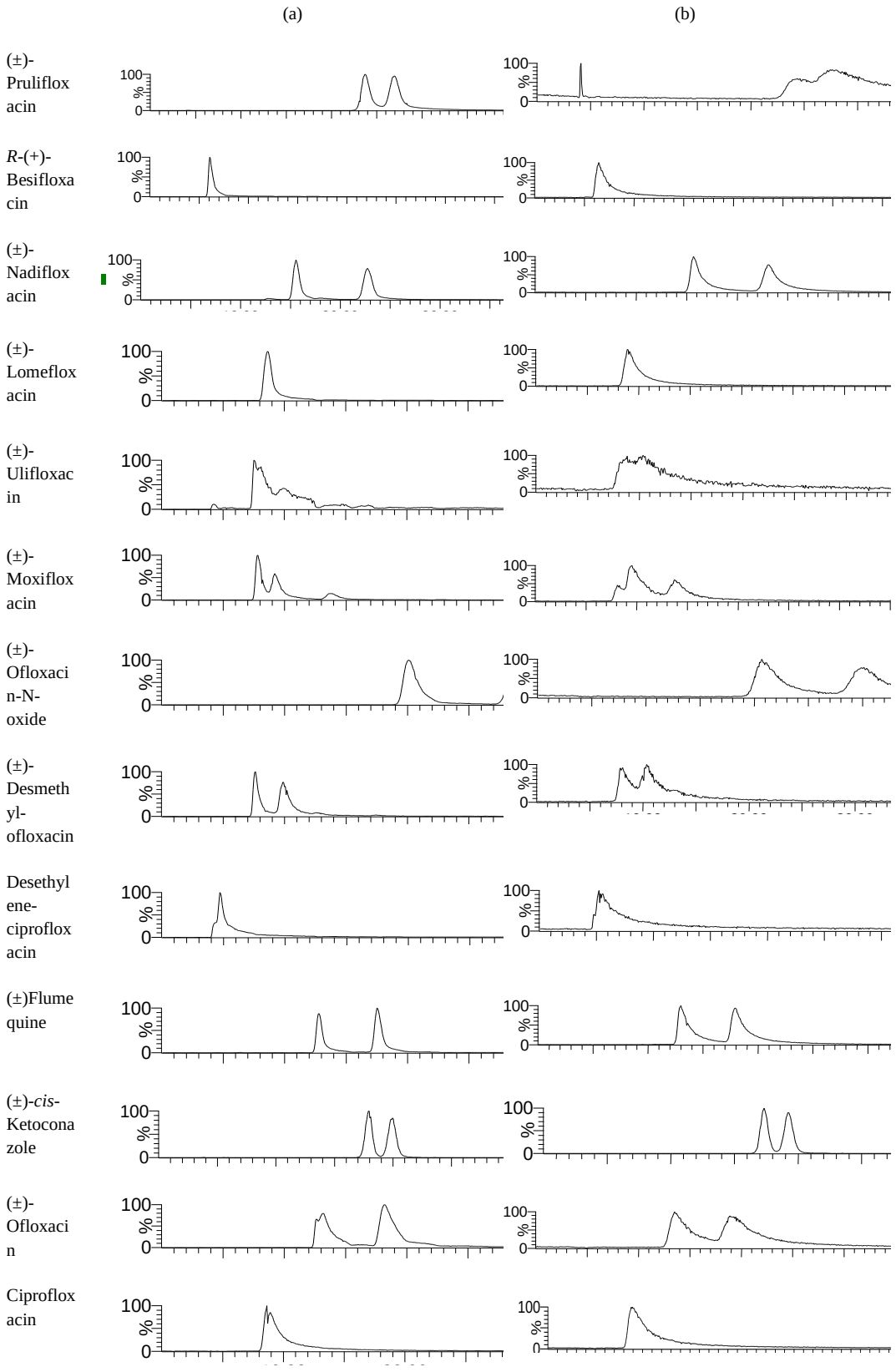
By comparing different flow rates, it was possible to conclude that higher enantioseparation was achieved at low flow rates. In this case, the best flow rate was found at 0.1 mL $min^{-1}$ (Figure S3a)., The addition of acid in the mobile phase, with consequent lower pH values reached, did not provide any enhancement in terms of separation for the majority of compounds (Figure S3b). Moreover, the salt concentration was another factor that played a key role in the enantioseparation as it modulated the interactions between analytes and the stationary phase. In this study, ammonium formate was considered. The study on the impact of the percentage of additive added revealed that higher ammonium formate percentages corresponded to higher retention of the analytes in the column along with an improved enantioseparation (Figure S3c).

As a result of these findings, the best chiral recognition for the widest group of analytes with a run time of only 40 minutes was achieved using a mobile phase composed of 10 mM ammonium formate/MeOH 1:99 (pH 6.4).

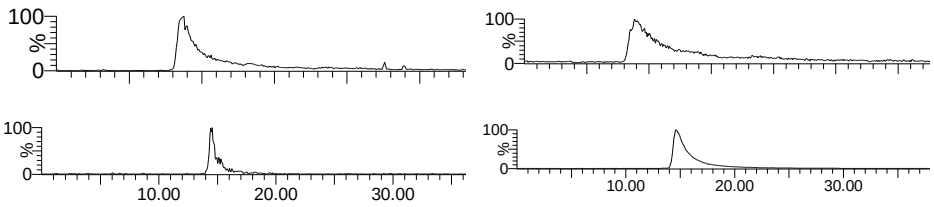
### 6.4.3 Method validation for the detection of quinolones and an antifungal drug in wastewater

The developed chiral method enabled the identification and quantification of all studied analytes with satisfactory sensitivity and specificity. Figure 6-2 shows the comparison of the mass chromatograms of MRM 1 transitions used for quantification purposes for each analyte between the standard solution in mobile phase and a spiked influent wastewater sample at a concentration of 100 ng $L^{-1}$.
Concentrations of compounds were calculated using the standard calibration curves which were built using ILIS approach. In general, calibration curves fitted 1/x. The mean correlation coefficients ($R^2$) of the calibration curves were on average > 0.99 for the investigated compounds (Table 6-3). The linearity ranges varied depending on the analyte. The lowest IDL values were detected for nadifloxacin and flumequine.

**Figure 6-2** Chromatograms of the quantification MRM transition for each investigated analyte of (a) a standard solution and (b) a spiked influent wastewater sample at a concentration of 100 ng $L^{-1}$ with OZ-RH column.



Norfloxacin




Nalidixic acid

**Table 6-3** Validation parameters - retention time, relative retention time, linearity range, correlation coefficient obtained from calibration curve and instrumental and method limits of detection and instrumental and method limits of quantification.

| Compound | $R_t$ (min) | Rel. $R_t$ | Sample diluent | | | | WWTP influent | |
|---|---|---|---|---|---|---|---|---|
| | | | Linearity range (µg/L) | $R^2$ | $IDL_{S/N}$ (µg/L) | $IQL_{S/N}$ (µg/L) | MDL (ng/L) | MQL (ng/L) |
| Ciprofloxacin | 8.7 ± 0.1 | 2.5 | 0.05-1000 | 0.9945 | 0.050 | 0.100 | 0.6 | 1.1 |
| Desethylene-ciprofloxacin | 6.6 ± 1.1 | 1.3 | 5.0-1000 | 0.9906 | 5.000 | 5.000 | 54.3 | 54.3 |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 13.1 ± 0.1 | 0.2 | 0.25-1000 | 0.9983 | 0.250 | 0.250 | 2.2 | 2.2 |
| *R*-(+)-Ofloxacin | 18.3 ± 0.5 | 2.5 | 0.25-1000 | 0.9973 | 0.250 | 0.250 | 2.3 | 2.3 |
| Norfloxacin | 9.0 ± 0.3 | 4.1 | 0.25-1000 | 0.9900 | 0.250 | 5.000 | 3.1 | 62.6 |
| *S*-(-)-Ofloxacin-*N*-oxide | 20.3 ± 0.2 | 0.5 | 0.5-1000 | 0.9981 | 0.500 | 1.000 | 4.8 | 9.6 |
| *R*-(+)-Ofloxacin-*N*-oxide | 29.2 ± 0.5 | 1.8 | 0.5-1000 | 0.9974 | 0.500 | 1.000 | 5.5 | 10.9 |
| *S*-(-)-Desmethyl-ofloxacin | 7.8 ± 0.1 | 0.4 | 0.125-1000 | 0.9985 | 0.125 | 0.500 | 1.2 | 5.0 |
| *R*-(+)-Desmethyl-ofloxacin | 9.9 ± 0.1 | 0.4 | 0.125-1000 | 0.9982 | 0.125 | 0.500 | 1.3 | 5.1 |
| Nalidixic acid | 14.5 ± 0.1 | 2.9 | 0.01-2000 | 0.9940 | 0.010 | 0.025 | 0.1 | 0.3 |
| (±)-Lomefloxacin | 8.8 ± 0.1 | 2.6 | 0.25-2000 | 0.9981 | 0.250 | 0.250 | 2.6 | 2.6 |
| *R,R*-Moxifloxacin | 8.3 ± 0.1 | 0.7 | 0.5-1000 | 0.9902 | 0.500 | 0.500 | 4.2 | 4.2 |
| *S,S*- Moxifloxacin | 9.0 ± 0.1 | 0.6 | 0.5-1000 | 0.9914 | 0.500 | 0.500 | 6.4 | 6.4 |
| Moxifloxacin-N-sulphate | 13.6 ± 0.2 | 1.7 | 0.5-2000 | 0.9941 | 0.500 | 1.000 | 5.7 | 11.3 |
| Prulifloxacin-E1 | 23.4 ± 0.9 | 2.4 | 0.5-1000 | 0.9969 | 0.500 | 0.500 | 5.8 | 5.8 |
| Prulifloxacin-E2 | 26.5 ± 0.5 | 2.5 | 0.5-1000 | 0.9966 | 0.500 | 0.500 | 5.1 | 5.1 |
| Ulifloxacin-E1 | 9.0 ± 0.6 | 6.1 | 2.5-1000 | 0.9981 | 2.500 | 2.500 | 23.5 | 23.5 |
| Ulifloxacin-E2 | 11.2 ± 0.9 | 7.8 | 2.5-1000 | 0.9950 | 2.500 | 2.500 | 33.9 | 33.9 |
| (±)-*cis*-Ketoconazole-E1 | 16.3 ± 0.1 | 0.4 | 0.05-1000 | 0.9986 | 0.050 | 0.050 | 0.5 | 0.5 |
| (±)-*cis*-Ketoconazole-E2 | 18.1 ± 0.1 | 2.1 | 0.05-1000 | 0.9976 | 0.050 | 0.050 | 0.7 | 0.7 |
| Flumequine-E1 | 12.9 ± 0.1 | 0.2 | 0.025-1000 | 0.9991 | 0.025 | 0.500 | 0.3 | 5.3 |
| Flumequine-E2 | 17.5 ± 0.1 | 0.1 | 0.025-1000 | 0.9978 | 0.025 | 0.500 | 0.3 | 5.3 |
| Nadifloxacin-E1 | 15.2 ± 0.1 | 0.3 | 0.025-1000 | 0.9989 | 0.025 | 0.500 | 0.2 | 4.3 |
| Nadifloxacin-E2 | 22.4 ± 0.2 | 0.2 | 0.025-1000 | 0.9978 | 0.025 | 0.500 | 0.2 | 5.0 |
| *R*-(+)-Besifloxacin | 6.4 ± 0.2 | 3.6 | 1.0-1000 | 0.9916 | 1.000 | 1.000 | 11.9 | 11.9 |

Overall, most analytes showed good linearity ranges, from 0.025 µg $L^{-1}$ up to 1000 or 2000 µg $L^{-1}$ (for single enantiomer or racemate respectively). Nalidixic acid showed the lowest IQL (0.025) (Table 6-3). Good enantiomeric resolution ($R_s \geq 1.0$), allowing for quantification of individual enantiomers was obtained for six analytes (Table 6-4). Since (±)-desmethyl-ofloxacin and (±)-ulifloxacin showed lower enantiomeric resolution, results for their single enantiomers should be considered on a semi-quantitative basis. EF values, obtained from racemate standard solutions injected across three concentration ranges, were on average 0.50 and were reproducible (Table 6-4).

**Table 6-4** Validation parameters - enantiomeric fraction (EF) or diastereomeric fraction (DF) and enantiomeric resolution (Rs) of compounds, which stereoisomers were separated under studied conditions, in mobile phase (MP) and in wastewater (WW).

| **Compounds** | **Rs** | | **EF (n=9)** | | |
|---|---|---|---|---|---|
| | **In MP** | **In WW** | **5 µg/L** | **50 µg/L** | **500 µg/L** |
| (±)-Ofloxacin | 1.25 | 0.89 | 0.53±0.01 | 0.49±0.01 | 0.49±0.00 |
| (±)-Ofloxacin-*N*-oxide | 1.71 | 1.07 | 0.49±0.01 | 0.48±0.01 | 0.50±0.01 |
| (±)-Desmethyl-ofloxacin | 0.97 | 0.56 | 0.50±0.04 | 0.51±0.02 | 0.51±0.01 |
| (±)-Prulifloxacin | 1.06 | 0.46 | 0.49±0.04 | 0.41±0.01 | 0.47±0.02 |
| (±)-Ulifloxacin | 0.67 | 0.41 | 0.51±0.01 | 0.49±0.01 | 0.49±0.01 |
| (±)-Flumequine | 1.91 | 1.10 | 0.51±0.03 | 0.50±0.02 | 0.49±0.02 |
| (±)-Nadifloxacin | 2.86 | 1.44 | 0.51±0.02 | 0.52±0.01 | 0.50±0.02 |
| (±)-*cis*-Ketoconazole- | 1.20 | 1.08 | 0.51±0.01 | 0.51±0.01 | 0.50±0.01 |
| **Compound** | **Rs** | | **DF (n=9)** | | |
| | **In MP** | **In WW** | **5 µg/L** | **50 µg/L** | **500 µg/L** |
| (±)-Moxifloxacin | 0.84 | 0.21 | 0.53±0.03 | 0.50±0.04 | 0.51±0.01 |

MDL and MQL ranged from 0.1 (nalidixic acid) to 54.3 ng $L^{-1}$ (desethylene ciprofloxacin) and from 0.3 to 54.3 ng $L^{-1}$ (Table 6-3). The instrumental and method precision was on average <20% (Tables 6-5 and S3). Quantification can be misinterpreted by matrix effects, especially when LC-(ESI) MS/MS are used for complex matrices. Indeed, ME results can be interpreted as signal enhancement over 100% and as signal suppression below 100%. Ion suppression determined by calculations without ILIS response showed how the presence of ILIS well compensated the ion suppression in the matrix, even for those compounds that had not their corresponding isotopically labelled or deuterated analogue. (Table 6-6). HLB cartridges were chosen as sorbents of choice for chiral separations of the drugs investigated with OZ-RH column. Abs recovery and relative recoveries data are reported in Table 6-6. Recoveries were between 70% and 120% for all analysed compounds. Relative SPE recovery and ME showed the ability of ILIS to correct and compensate for the complexity of the matrix.

**Table 6-5** Validation parameters - method precision

| **Analytes** | **Intra-day RSD% (n=4)** | | | | | | | | | **Inter-day RSD% (n=3)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 ng/L** D 1* | 25 ng/L D 2 | 25 ng/L D 3 | 250 ng/L D 1 | 250 ng/L D 2 | 250 ng/L D 3 | 2500 ng/L D 1 | 2500 ng/L D 2 | 2500 ng/L D 3 | 25 ng/L | 250 ng/L | 2500 ng/L |
| Ciprofloxacin | 13.6 | 1.8 | 1.4 | 11.9 | 14.7 | 9.4 | 12.0 | 8.0 | 6.2 | 5.6 | 12.0 | 8.7 |
| Desethylene-ciprofloxacin | 13.4 | 0.8 | 13.0 | 3.9 | 7.1 | 1.3 | 14.5 | 8.9 | 7.6 | 9.1 | 4.1 | 10.3 |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 9.0 | 15.5 | 2.3 | 1.8 | 3.5 | 4.8 | 0.5 | 2.7 | 0.4 | 8.9 | 3.4 | 1.2 |
| *R*-(+)-Ofloxacin | 10.4 | 9.6 | 7.2 | 4.6 | 3.0 | 2.5 | 6.2 | 3.2 | 4.9 | 9.1 | 3.4 | 4.8 |
| Norfloxacin | 5.0 | 0.0 | 14.1 | 14.9 | 17.2 | 15.6 | 10.3 | 7.6 | 18.6 | 6.4 | 15.9 | 12.2 |
| *S*-(-)-Ofloxacin-*N*-oxide | 16.0 | 7.8 | 4.3 | 9.3 | 7.0 | 0.2 | 2.1 | 5.0 | 3.9 | 9.4 | 5.5 | 3.7 |
| *R*-(+)-Ofloxacin-*N*-oxide | 6.0 | 15.6 | 8.2 | 3.8 | 2.9 | 1.3 | 6.6 | 3.2 | 2.5 | 9.9 | 2.7 | 4.1 |
| *S*-(-)-Desmethyl-ofloxacin | 3.4 | 2.7 | 8.8 | 5.1 | 4.9 | 5.1 | 2.0 | 2.3 | 0.8 | 5.0 | 5.0 | 1.7 |
| *R*-(+)-Desmethyl-ofloxacin | 3.5 | 17.1 | 10.1 | 1.8 | 3.2 | 4.5 | 0.9 | 2.1 | 3.0 | 10.2 | 3.2 | 2.0 |
| Nalidixic acid | 2.9 | 0.4 | 2.9 | 7.6 | 9.5 | 12.2 | 11.4 | 7.6 | 17.9 | 2.1 | 9.8 | 12.3 |
| (±)-Lomefloxacin | 5.2 | 14.1 | 14.1 | 2.5 | 2.7 | 3.3 | 1.8 | 3.8 | 13.0 | 11.1 | 2.8 | 6.2 |
| *R,R*-Moxifloxacin | 3.6 | 17.4 | 16.8 | 10.5 | 13.6 | 9.4 | 8.0 | 8.0 | 0.9 | 12.6 | 11.2 | 5.6 |
| *S,S*- Moxifloxacin | 11.7 | 1.9 | 9.2 | 9.3 | 2.5 | 0.4 | 7.2 | 1.7 | 3.5 | 7.6 | 4.1 | 4.1 |
| Moxifloxacin-*N*-sulphate | 8.3 | 3.2 | 13.8 | 5.1 | 5.4 | 7.3 | 6.2 | 3.5 | 1.6 | 8.4 | 5.9 | 3.8 |
| Prulifloxacin-E1 | 4.5 | 14.6 | 18.9 | 4.1 | 2.2 | 2.9 | 8.3 | 4.4 | 4.6 | 12.7 | 3.1 | 5.8 |
| Prulifloxacin-E2 | 7.6 | 17.7 | 20.2 | 5.4 | 0.7 | 4.7 | 9.7 | 2.6 | 2.6 | 15.2 | 3.6 | 5.0 |
| Ulifloxacin-E1 | 17.5 | 3.2 | 11.4 | 12.1 | 15.8 | 15.8 | 2.1 | 10.1 | 7.1 | 10.7 | 14.6 | 6.4 |
| Ulifloxacin-E2 | 11.9 | 10.2 | 1.6 | 12.2 | 10.6 | 1.9 | 1.3 | 2.9 | 6.4 | 7.9 | 8.2 | 3.5 |
| (±)-*cis*-Ketoconazole-E1 | 2.2 | 16.4 | 5.3 | 2.9 | 2.6 | 4.0 | 2.3 | 2.9 | 2.1 | 8.0 | 3.2 | 2.4 |
| (±)-*cis*-Ketoconazole-E2 | 2.2 | 5.0 | 5.7 | 4.9 | 1.6 | 1.9 | 2.8 | 2.8 | 1.1 | 4.3 | 2.8 | 2.2 |
| Flumequine-E1 | 1.3 | 3.2 | 3.0 | 1.2 | 0.6 | 0.9 | 0.2 | 2.0 | 1.0 | 2.5 | 0.9 | 1.1 |
| Flumequine-E2 | 2.3 | 4.6 | 0.0 | 1.8 | 3.4 | 0.1 | 2.6 | 4.4 | 6.6 | 2.3 | 1.8 | 4.5 |
| Nadifloxacin-E1 | 1.6 | 3.4 | 9.5 | 5.1 | 0.5 | 0.2 | 6.2 | 2.4 | 1.3 | 4.8 | 1.9 | 3.3 |
| Nadifloxacin-E2 | 5.8 | 5.5 | 12.6 | 5.9 | 5.0 | 1.5 | 2.9 | 1.1 | 13.8 | 8.0 | 4.1 | 5.9 |
| *R*-(+)-Besifloxacin | 7.2 | 5.6 | 9.6 | 8.0 | 7.6 | 11.6 | 6.4 | 3.7 | 9.7 | 7.5 | 9.1 | 6.6 |

*-D indicates day

**- the following concentrations were used: 10, 100 and 1000 ng $L^{-1}$ in the case of compounds that were not enantioseparated

**Table 6** Matrix effect, absolute and relative SPE recoveries for the studied analytes

| | %ME | | Abs | SPE relative recovery % (n=3) | | |
|---|---|---|---|---|---|---|
| **Analyte** | With ILIS | Without ILIS | **recovery %** | 25 ng/L* | 250 ng/L* | 2500 ng/L* |
| Ciprofloxacin | 117.0 | 46.8 | 68.2 | 84.3 ± 5.7 | 83.8 ± 1.7 | 101.7 ± 31.7 |
| Desethylene-ciprofloxacin | 74.6 | 23.1 | 40.3 | 83.8 ± 8.9 | 107.7 ± 3.5 | 84.9 ± 4.5 |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 111.6 | 81.2 | 114.8 | 110.8 ± 9.3 | 113.6 ± 1.5 | 111.9 ± 1.1 |
| *R*-(+)-Ofloxacin | 107.6 | 78.3 | 141.5 | 113.8 ± 1.9 | 98.9 ± 1.8 | 106.4 ± 2.5 |
| Norfloxacin | 79.2 | 15.4 | 271.7 | 73.0 ± 3.7 | 82.0 ± 1.0 | 84.7 ± 2.6 |
| *S*-(-)-Ofloxacin-*N*-oxide | 108.7 | 79.0 | 124.3 | 103.1 ± 5.1 | 106.7 ± 6.2 | 103.0 ± 2.1 |
| *R*-(+)-Ofloxacin-*N*-oxide | 102.9 | 75.1 | 168.0 | 82.2 ± 11.9 | 95.2 ± 3.0 | 96.7 ± 2.0 |
| *S*-(-)-Desmethyl-ofloxacin | 96.3 | 37.6 | 67.8 | 97.1 ± 9.1 | 103.1 ± 3.5 | 101.8 ± 3.9 |
| *R*-(+)-Desmethyl-ofloxacin | 95.6 | 32.0 | 129.8 | 111.3 ± 11.7 | 88.8 ± 2.9 | 92.4 ± 2.2 |
| Nalidixic acid | 98.6 | 63.8 | 112.6 | 89.8 ± 7.9 | 89.3 ± 10.8 | 90.1 ± 12.7 |
| (±)-Lomefloxacin | 90.8 | 36.1 | 99.6 | 102.9 ± 6.0 | 90.4 ± 1.0 | 97.6 ± 1.2 |
| *R,R*-Moxifloxacin | 104.0 | 86.3 | 70.5 | 118.0 ± 0.8 | 118.7 ± 1.4 | 117.3 ± 0.9 |
| *S,S*- Moxifloxacin | 90.0 | 61.1 | 87.9 | 78.4 ± 5.6 | 71.7 ± 6.9 | 85.7 ± 7.5 |
| Moxifloxacin-*N*-sulphate | 116.4 | 45.5 | 88.8 | 85.2 ± 4.0 | 82.5 ± 2.8 | 96.9 ± 3.6 |
| Prulifloxacin-E1 | 109.0 | 132.4 | 73.5 | 73.3 ± 2.8 | 81.9 ± 8.2 | 105.1 ± 5.7 |
| Prulifloxacin-E2 | 102.2 | 74.6 | 144.4 | 97.8 ± 20.4 | 96.8 ± 3.8 | 100.7 ± 3.4 |
| Ulifloxacin-E1 | 119.1 | 47.8 | 92.5 | 119.0 ± 0.5 | 98.6 ± 3.5 | 101.1 ± 4.5 |
| Ulifloxacin-E2 | 73.8 | 20.8 | 274.1 | 80.7 ± 9.3 | 70.3 ± 0.3 | 70.5 ± 0.5 |
| (±)-*cis*-Ketoconazole-E1 | 95.5 | 69.4 | 120.3 | 104.8 ± 1.7 | 107.3 ± 3.6 | 106.4 ± 0.8 |
| (±)-*cis*-Ketoconazole-E2 | 95.8 | 69.7 | 112.1 | 72.0 ± 4.2 | 70.0 ± 2.2 | 71.0 ± 1.6 |
| Flumequine-E1 | 98.3 | 66.5 | 136.8 | 90.2 ± 2.7 | 95.0 ± 1.1 | 95.9 ± 0.1 |
| Flumequine-E2 | 97.8 | 70.8 | 155.6 | 89.3 ± 1.8 | 96.4 ± 0.3 | 98.4 ± 0.9 |
| Nadifloxacin-E1 | 112.3 | 75.9 | 119.3 | 118.3 ± 0.6 | 115.6 ± 4.5 | 116.8 ± 3.0 |
| Nadifloxacin-E2 | 111.7 | 80.9 | 136.0 | 94.9 ± 6.8 | 98.6 ± 4.7 | 107.0 ± 4.6 |
| *R*-(+)-Besifloxacin | 85.6 | 17.4 | 221.6 | 106.8 ± 2.7 | 73.5 ± 5.7 | 72.0 ± 1.6 |

*- the following concentrations were used: 50, 500 and 5000 ng $L^{-1}$ in the case of compounds that were not enantioseparated

### 6.4.4 Application of the method for the analysis of influent wastewater samples

The developed and validated method was applied in a one-week monitoring campaign from a wastewater treatment plant serving a large city in the South West of the UK. As shown in the results (Table 6-7), ciprofloxacin was the target drug with the highest concentrations found, especially at the beginning of the week. Its metabolite desethylene-ciprofloxacin was detected at quantifiable concentrations in two samples (just above the MQL), whilst it was present at <MQL across the week due to its high MQL value. A predominance of the *S*-(-)-ofloxacin (*L*-ofloxacin) in terms of concentrations was detected with respect to *R*-(+)-ofloxacin giving a constant ratio 3:1 over the week.

A confirmation of their ratio can be explained only through calculation of their mass loads. This is the first time that a complete investigation of ofloxacin metabolic profile was detected in influent wastewater in the UK. Their metabolic pattern was reflected by the presence of their metabolites: (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin. Unfortunately, in this case a ratio could not be calculated as they were present at <MQL. Other two chiral drugs were found: (±)-*cis*-ketoconazole and (±)-flumequine. Interestingly, (±)-*cis*-ketoconazole was found enriched of the first eluting isomer, which suggests potential enantioselective metabolism. It should be noted that Hamdy and Brocks [50] found evidence of non-linear stereoselective pharmacokinetics in rats, whilst no stereoselective metabolism by liver microsomes. Hence, further work is needed to support this finding. This was the first time that the enantiomers of (±)-prulifloxacin and (±)-ulifloxacin were investigated, even though their detection was not expected to be found in wastewater in the UK. For this reason, the study on their enantiomeric profiling was not possible. In the developed method, the enantiomers of (±)-lomefloxacin were not separated, so the evaluation of its enantiomeric profiling was not possible. The following achiral compounds were also present: norfloxacin and nalidixic acid.

Overall, these results showed constant concentrations across the sampling week for all the detected and quantifiable analytes. These results will be used in a further study to estimate drug use via WBE.

**Table 6-7** Concentration of targeted compounds in wastewater samples during one week monitoring campaign in the South West of the UK.

| **Analyte** | **Concentration [ng $L^{-1}$]** | | | | | | |
|---|---|---|---|---|---|---|---|
| | Monday | Tuesday | Wednesday | Thursday | Friday | Saturday | Sunday |
| Ciprofloxacin | 175 ± 10 | 192 ± 7 | 197 ± 6 | 149 ± 10 | 124 ± 6 | 144 ± 2 | 144 ± 5 |
| Desethylene-ciprofloxacin | 61 ± 2 | 58 ± 1 | < MQL | < MQL | < MQL | < MQL | < MQL |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 50 ± 1 | 38 ± 6 | 43 ± 2 | 32 ± 2 | 31 ± 1 | 46 ± 3 | 45 ± 2 |
| *R*-(+)-Ofloxacin | 13 ± 1 | 12 ± 1 | 15 ± 1 | 10 ± 1 | 10 ± 1 | 14 ± 3 | 14 ± 3 |
| Norfloxacin | n.d. | n.d. | n.d. | < MQL | < MQL | n.d. | < MQL |
| *S*-(-)-Ofloxacin-*N*-oxide | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL |
| *R*-(+)-Ofloxacin-*N*-oxide | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL |
| *S*-(-)-Desmethyl-ofloxacin | 12 ± 4 | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL |
| *R*-(+)-Desmethyl-ofloxacin | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL | < MQL |
| Nalidixic acid | 5 ± 1 | 2 ± 1 | 1 ± 0 | < MQL | 1 ± 0 | 1 ± 0 | 1 ± 0 |
| (±)-Lomefloxacin | 3 ± 1 | 3 ± 1 | < MQL | < MQL | 3 ± 0 | < MQL | < MQL |
| *R,R*-Moxifloxacin | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *S,S*- Moxifloxacin | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Moxifloxacin-*N*-sulphate | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Prulifloxacin-E1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Prulifloxacin-E2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Ulifloxacin-E1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Ulifloxacin-E2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| (±)-*cis*-Ketoconazole-E1 | 8 ± 1 | 9 ± 2 | 10 ± 1 | 7 ± 1 | 6 ± 1 | 9 ± 1 | 11 ± 1 |
| (±)-*cis*-Ketoconazole-E2 | 5 ± 1 | 5 ± 1 | 6 ± 0 | 4 ± 0 | 4 ± 0 | 6 ± 0 | 6 ± 0 |
| Flumequine-E1 | < MQL | < MQL | < MQL | n.d. | < MQL | < MQL | < MQL |
| Flumequine-E2 | n.d. | < MQL | n.d. | n.d. | n.d. | < MQL | < MQL |
| Nadifloxacin-E1 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Nadifloxacin-E2 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| *R*-(+)-Besifloxacin | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

n.d. - not detected

## 6.5 Conclusions

Monitoring activity of antibiotic consumption is crucial for public health monitoring, especially when the spreading of antibiotic resistance is related to an extensive use. One of the antibiotic class that has a rising concern in antibiotic resistance is the quinolones. A near real-time monitoring tool is provided by WBE that can also allow for the determination of quinolones consumption biomarkers via analysis of human urinary metabolites. Since many quinolones are distributed as racemates, pharmacokinetics and pharmacodynamics differences occur in humans and animals, and their effects can be found in the environment. However, analytical methods that enable the simultaneous determination of quinolones biomarkers along with their chiral composition are missing. To aid enantiomeric profiling of quinolones and an antifungal drug in WBE, a novel chiral analytical method based on SPE with HLB followed by enantioselective HPLC-MS/MS with the usage of an OZ-RH column was developed and fully validated in wastewater. This method showed very good performance: >70% SPE recoveries, very good sensitivity (MDLs and MQLs at ppt levels), high linearity range and method precision < 20%. It allowed for the analysis of 16 human and veterinary quinolones drugs as potential biomarkers in wastewater. This method was applied in a one week monitoring campaign of a large wastewater treatment plant in the South of the UK, where many targeted drugs were found at quantifiable concentrations. When these targeted drugs were detected, their concentrations did not vary over the week.

Enantiomeric profiling study revealed that (±)-ofloxacin was found enriched with *S*-(-)-enantiomer, probably due to higher consumption of the enantiomerically pure drug. The detection of (±)-ofloxacin metabolites in wastewater indicated that their presence was due to their human origin and, thus, to the ofloxacin consumption. A slightly enrichment of the first-eluting enantiomer was also observed in the case of (±)-*cis*-ketoconazole, even though further investigation is needed. To the authors' knowledge, this is the first time that chiral separation in reverse phase LC-MS was simultaneously undertaken for (±)-ofloxacin with its main metabolites (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin, (±)-moxifloxacin, the prodrug (±)-prulifloxacin with its active compound (±)-ulifloxacin, (±)-*cis*-ketoconazole, (±)-flumequine, (±)-nadifloxacin and *R*-(+)-besifloxacin and their enantiomeric profiling was investigated at

enantiomeric level in composite wastewater samples. As achiral quinolones were also included, the method is suitable for monitoring purposes.

## 6.6 Supplementary Data

The following supplementary data are contained in Appendix 4:

Table S1 Selected analytes and their properties (MW=molecular weight).

Table S2 Studied mobile phase compositions with CHIRALCEL® OZ-RH column.

Table S3 Validation parameters -instrumental precision

Figure S1 CHIRALCEL® OZ-RH column – impact of the organic content on the separation of studied analytes (mobile phases in the legend are referred to the organic modifier mentioned in the title of the graphic).

Figure S2 CHIRALCEL® OZ-RH column – (a) impact of the organic modifiers used on the separation of studied analytes. The mobile phase composition was constituted by the organic solvent specified in the legend and by 5% of 5mM ammonium acetate as aqueous content; (b) Impact of the nature of the organic content in the mobile phase on enantiomeric resolution. The mobile phases considered were the best performing: 95:5 5mM ammonium formate for IPA and ACN, 99:1 5mM ammonium formate for EtOH, 99:1 10mM ammonium formate for MeOH.

Figure S3 CHIRALCEL® OZ-RH column – impact of (a) the flow rate, (b) pH and (c) percentage of the additive content on the separation of studied analytes. The mobile phase used for the first graphic was made of acetonitrile:water 95:5 5mM ammonium acetate.

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# Chapter 7: Enantiomeric profiling of quinolones and monitoring of resistance genes in European wastewaters.

## 7.1 Summary

The misuse of quinolones and fluoroquinolones represents an increasing concern especially due to the spreading of antibacterial resistance. Real-time monitoring of antibiotics is, therefore, essential to verify (inappropriate and unnecessary) use by humans and in veterinary medicine. Along with surveillance and statistics data, a helpful on-time monitoring tool is represented by WBE through the identification of biomarkers of exposure to antibiotics. Discussed here is the first pan-European study on enantiomeric profiling and monitoring of chiral quinolones in wastewater. This study identified several new potential biomarkers for quinolones consumption to be used in WBE context. They were: (±)-ofloxacin with its main metabolites (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin, (±)-

moxifloxacin, the precursor (±)-prulifloxacin with its active compound (±)-ulifloxacin, (±)-*cis*-ketoconazole (the only antifungal included), (±)-flumequine, (±)-nadifloxacin and *R*-(+)-besifloxacin. The investigation of loads of chiral quinolones enantiomers in wastewater and their correlation with their human stereoselective metabolism enabled to find out areas where consumption and direct disposal of ciprofloxacin and ofloxacin occurred (i.e. southern European locations). In northern European cities, *S*-(-)-ofloxacin loads were predominant with respect to *R*-(+)-ofloxacin. Enantiomerically pure *S*-(-)-ofloxacin (levofloxacin) was detected in wastewaters from southern European cities, showing a stereoselective usage of the drug. Nalidixic acid, norfloxacin and lomefloxacin were founds at low population-normalised loads. Diastereomerically and enantiomerically *S,S*-moxifloxacin and *S,S*-moxifloxacin-*N*-sulphate were detected in wastewater presumably due to metabolism of moxifloxacin. For the first time, population-normalised ulifloxacin loads were found in Milan and Castellon with average values at 22.3 and 1.5 mg $day^{-1}$ 1000 $people^{-1}$ respectively as a result of prulifloxacin metabolism. No racemic prulifloxacin was detected as a consequence of its direct disposal. (±)-*cis*-Ketoconazole was enriched with the first-eluting enantiomer, thus suggesting potential enantioselective metabolism. Enrichment of flumequine with first-eluting enantiomer in all the samples was explained as a result of animals' metabolism rather than its direct disposal. Moreover, the occurrence of fluoroquinolone resistance genes was investigated in wastewaters from several European locations. Interpretation of data was controversial even though the approach based on analytical and bioanalytical techniques was a promising means for further studies.

## 7.2 Introduction

Microorganisms, antimicrobial agents and the geographical region are three variables that play a key role in the occurrence of antimicrobial resistance in Europe [1]. Geographical gradients, from northern to southern Europe and from northern to eastern Europe, were reported for some combinations of antimicrobial agents and microorganisms [1]. As geographical differences in antibiotics prescription could be the evidence of a different consumption pattern and environmental occurrence, it is important to investigate the antimicrobial use and correlate their presence in the environment with the occurrence of antimicrobial resistance. Whilst data on

antimicrobial agents' usage are annually collected at a community and hospital level and are provided by the European Surveillance of Antimicrobial Consumption Network (ESAC-Net), data on antimicrobial resistance are given by the European Antimicrobial Resistance Surveillance Network (EARS-Net). These monitoring activities are therefore essential for delineating common and prompt directions and strategies in order to inform the policy's makers on antimicrobial resistance problems, which require international cooperation. These surveillance systems rely on national sales, reimbursement data (or a combination of them) and information taken from national drug registers. Unfortunately, a temporal delay of one year (or two years) usually occurs since epidemiological data are published. This may affect the efficiency of decision-making strategies. A promising monitoring tool, recently applied to another class of compounds (i.e. illicit drugs), is based on the WBE approach. Since humans can be considered as a source of both antibiotics and antibiotic resistance genes [2], they can release them as products of urinary excretion in the sewer system. Not all of these excretion products, so-called biomarkers, are suitable indicators of human antibiotic intake as some prerequisites need to be satisfied. A likely drug biomarker for WBE purposes needs to have also good stability in wastewater. A number of studies assessed stability of the targeted compounds in several environmental matrices, albeit without considering their stereoselectivity. As reported by Kummerer [3], fluoroquinolones are insensitive to hydrolysis and elevated temperatures, although they are degraded by UV light. Indeed, their stability in wastewater and during wastewater treatment is a concern, as these potent chemicals can be found in wastewater and then in receiving environment, thus contributing to antimicrobial resistance (AMR).

A number of studies reported antibiotics in the environmental matrices, especially because of their proved risk to the environmental and human health [3]. Others demonstrated that resistance genes spreading into the environment demonstrate an emergent issue [4, 5]. Only a few reports correlated antibiotic loads to the presence of antibiotic resistance gene [2]. None of them investigated antibiotic profiling focussing on both metabolic profile and stereochemistry dimension. There is therefore a knowledge gap regarding antibiotics in the environment and their impacts on AMR.

Therefore, the aim of this chapter is to:

(i) study daily loads of selected antibiotics in wastewaters from several European locations, which are known to have increasing resistance problem;

(ii) study the enantiomeric profiling of antibiotics;

(iii) evaluate the level of its antibiotic gene resistance in the wastewater;

(iv) analyse quinolones and gene resistance loads in the monitored areas.

## 7.3 Experimental

### 7.3.1 Chemicals and materials

The following analytes were selected for the study (Figure 7-1): (±)-ofloxacin, *S*-(-)-ofloxacin, (±)-ofloxacin-*N*-oxide, (±)-desmethyl-ofloxacin, (±)-lomefloxacin, (±)-prulifloxacin, (±)-ulifloxacin, (±)-ketoconazole, (±)-flumequine, (±)-nadifloxacin, *R,R*-moxifloxacin, *S,S*-moxifloxacin and *S,S*-moxifloxacin-N-sulphate and *R*-(+)-besifloxacin. The following deuterated and isotopic analogues of target analytes were used as ILIS: ciprofloxacin-$D_8$, (±)-ofloxacin-$D_3$, (±)-desmethyl-ofloxacin-$D_8$ and (±)-flumequine$^{13}C_3$. Standard stock solutions were initially prepared at 1 mg $mL^{-1}$ by dissolving (±)-ofloxacin-$D_3$ and (±)-flumequine$^{13}C_3$ in acetonitrile and ciprofloxacin-$D_8$ and (±)-desmethyl-ofloxacin-$D_8$ in water. A mixture of them was finally prepared from stock solutions at 1 mg $L^{-1}$ by dilution with mobile phase and it was used for spiking samples. CAS number, molecular formula, molecular weight, $pK_a$ and supplier information for targeted analytes is given in Appendix 4 (see Table S1). All standards and internal standards were of the highest purity available (>97%). Stock and working solutions of standards were stored at -20° C. Methanol, ammonium formate and formic acid were purchased from Sigma Aldrich, UK. Ultrapure water was obtained from PURELAB UHQ-PS Unit (Elga, UK).

### 7.3.2 Wastewater sample collection and storage

24-hours composite wastewater influent samples were collected over a week in March 2015 from several wastewater treatment plants across Europe. The used sampling protocol is described elsewhere [6].

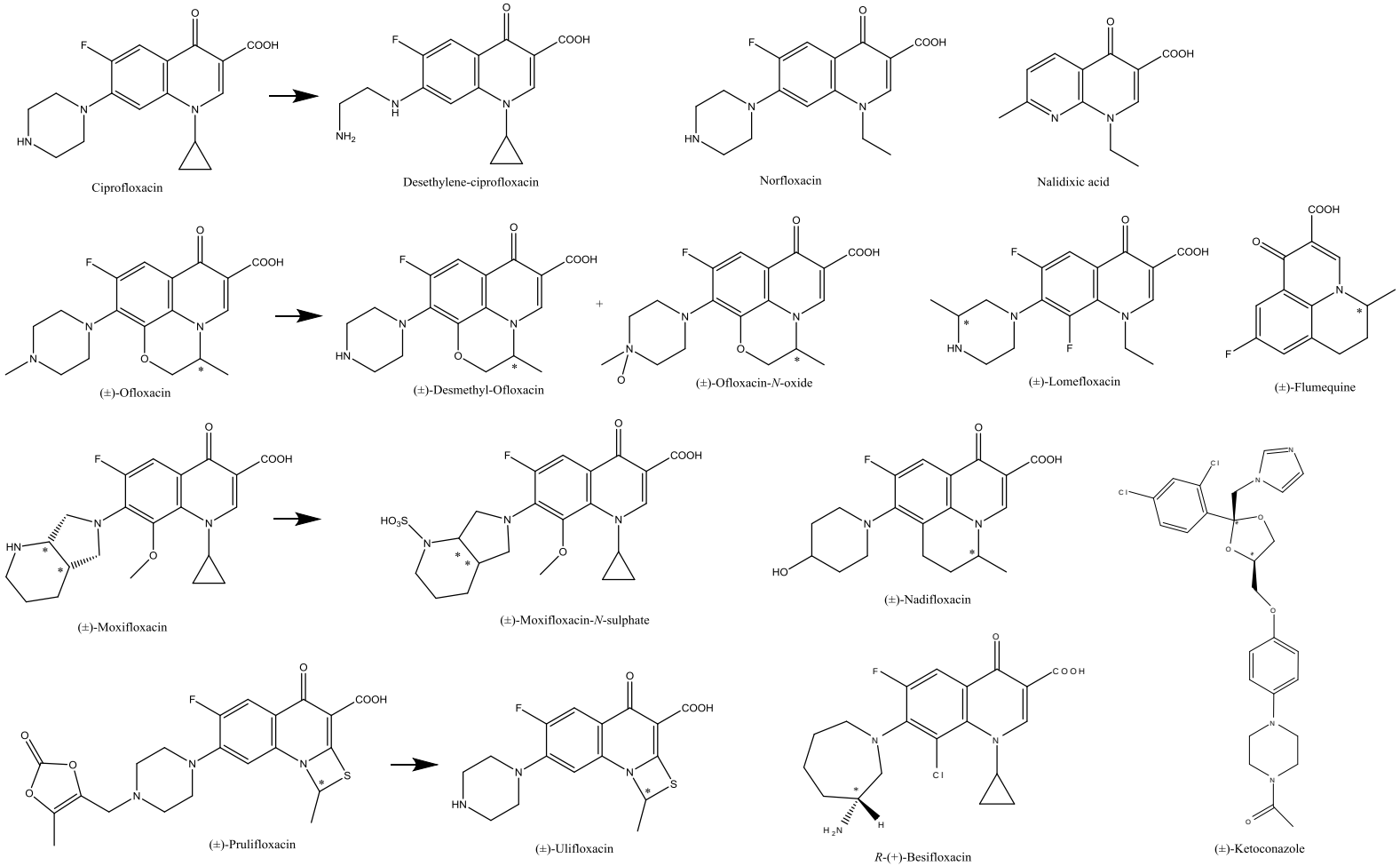



**Figure 7-1** Chiral and not chiral analytes selected in the study [(±)-cis-ketoconazole was the only antifungal included]. The arrow indicates that the produced analyte is a metabolite (to be noticed that not all the metabolites were included in the figure).

Sampling locations were in Norway (Oslo), United Kingdom (Bristol), Denmark (Lyngby), The Netherlands (Utrecht), Switzerland (Zurich), Italy (Milan) and Spain (Castellon) (Figure 7-2).

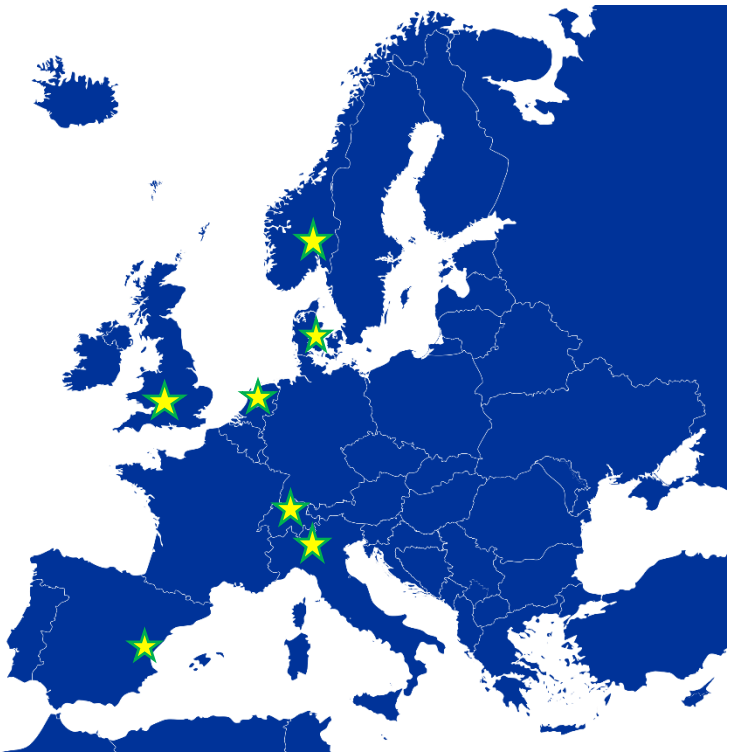


**Figure 7-2** Sampling locations.

Information on population and flow for cities involved in the study are provided in Table 7-1. Once collected, wastewater samples were transported to the local laboratory in refrigerated conditions and shipped on ice blocks to the UK within 24 hours.

**Table 7-1** Selected cities in the study, population and flow data.

| **City** | Bristol | Oslo | Milan | Utrecht | Castellon | Zurich | Lyngby |
|---|---|---|---|---|---|---|---|
| **Country** | UK | Norway | Italy | The Netherlands | Spain | Switzerland | Denmark |
| **Residential population** | 886650 | 580639 | 1100000 | 300000 | 180690 | 410000 | 531000 |
| **Day** | **Flow in $m^3$/day** | | | | | | |
| Monday | 197493 | 254570 | 597470 | 45970 | 37469 | 177167 | 148724 |
| Tuesday | 204491 | 252721 | 423110 | 44580 | 40476 | 160912 | 150936 |
| Wednesday | 198950 | 333480 | 403240 | 47740 | 50228 | 157084 | 147175 |
| Thursday | 197523 | 308279 | 412310 | 45030 | 49161 | 161005 | 144840 |
| Friday | 252682 | 277450 | 402240 | 49530 | 43728 | 161427 | 145197 |
| Saturday | 220687 | 256766 | 403020 | 46030 | 38301 | 200010 | 137793 |
| Sunday | 193194 | 250384 | 422690 | 46900 | 37243 | 243013 | 137244 |

### 7.3.3 Sample preparation and analysis with chiral HPLC-MS/MS

Samples were prepared by following a validated chiral analytical method for the detection and quantification of chiral and achiral quinolones and a chiral antifungal drug in wastewater as described in Chapter 6. Briefly, samples were filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK) and 50 mL were spiked with 50 µL of ILIS mixture at concentration of 1 mg $L^{-1}$. They were then solid-phase extracted by using Oasis HLB cartridges (60 mg, Waters, UK). Before the loading of the samples, these cartridges were conditioned with 3 mL of methanol and equilibrated with 3 mL of ultrapure water at a rate of 3 mL $min^{-1}$. The loading phase was at a rate of 8 mL $min^{-1}$. Washing step was carried out with 1 mL of ultrapure water at a rate of 3 mL $min^{-1}$, whilst the elution with 4 mL of methanol at a rate of 8 mL $min^{-1}$ into 5 mL silanised glass tubes. The extracts in the glass tubes were then evaporated to dryness under nitrogen flow (5-10 psi) by using a TurboVap evaporator (Caliper, UK). Reconstitution of the extracts was performed by adding 500 µL of the mobile phase, consisting of 10 mM ammonium formate/methanol 1:99 v/v with 0.05% formic acid. Before being transferred to polypropylene plastic vials bonded pre-slit PTFE/Silicone septa (Waters, UK), samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13mm). 20 µL were directly injected into a chiral HPLC-MS/MS. Samples from the monitoring campaign were prepared and analysed in duplicate.

The analysis was undertaken by using a Waters ACQUITY UPLC® system (Waters, Manchester, UK) with a chiral CHIRALCEL® OZ-RH column (5 µm particle size, L × I.D. 15 cm × 2.1 mm, Chiral Technologies, France) connected with a 2.0 mm × 2.0 mm guard filter (Chiral Technologies, France) in the column compartment (temperature set at 30°C). The autosampler was kept at 4°C. The flow rate was 0.1 mL $min^{-1}$ under isocratic conditions. A triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) equipped with an ESI was used in positive mode. Instrumental parameters are described in details in Chapter 6 (see paragraph 6.3.3). Data were acquired in MRM mode. Selected MRM transitions, CV and CE values for each compound were presented in Chapter 6 (see Table 6-2).

MassLynx 4.1 (Waters, UK) was used to control both systems, the Waters ACQUITY and the Xevo TQD. TargetLynx software (Waters, Manchester, UK) was used for data processing.

### 7.3.4 Sample preparation and analysis for *qnr* gene quantification

UK wastewater samples were firstly tested on non-selective media plates for proving the suitable volume to be used in a further qualitative test. Indeed, the test "dry run on non-selective media" was performed by using 100 µL and 200 µL of refrigerated wastewater samples (day $6^{th}$ and $7^{th}$). Plates were then incubated at 37° C overnight. As shown in Figure S1, plates with 100 µL of wastewater incubated provided a distinct bacteria growth. 100 µL of wastewater samples from all the European sites were therefore incubated in cysteine-, lactose-, and electrolyte-deficient (CLED) agar plates. CLED agar (Sigma Aldrich, UK) media was prepared as follows. 36.15 g of powder were weighted to make 1 L of CLED agar solution and transferred to a sterilised 1 L glass bottle. The bottle was filled with 1 L of distilled water. Once it was properly dissolved, it was placed in the autoclave for 20 minutes. The CLED agar was then finally poured in sterilised petri dishes. ~16 colonies from each plate were isolated and incubated singularly (an example is shown in Figure S2). Every single colony was stocked in cryogenic vials (2mL, Fisherbrand) containing 500 µL of 30% LB/Glycerol filter-sterilised and kept in the freezer -80°C as reference. The plates were stored in the fridge with paper-tape. Plates of the incubated wastewater are shown in Figure S3-4.

#### 7.3.4.1 DNA extraction

Triplicate wastewater samples of 1mL each were centrifuged for 5' at 3000 g and the cell pellet was resuspended in 200 µL PBS. 5 µL lysozyme were then added, followed by an incubation of 15' at 37 °C. 200 µL Binding buffer and 40 µL proteinase K were added and left in incubation for 10' at 70 °C. 100 µL IPA were added before the filtration in a filter tube and the centrifugation for 1' at 8000 g. The flow in the collecting tube was discarded and 500 µL Inhibitor removal buffer was added to the filter tube assembled in a new collecting tube. The sample was centrifuged for 1' at 8000 g and the flow was discarded. 500 µL washing buffer were added to the filter tube assembled in a new collecting tube. The sample was centrifuged for 1' at 8000 g and the flow was discarded. This step was repeated and, finally, the sample was centrifuged for 10' at 9000 g. The DNA elution was performed by adding 200 µL pre-warmed elution buffer into the High Pure Filter Tube. This sample preparation was followed the manufacturer's instructions (High Pure PCR Template Preparation Kit, Roche, Germany). DNA concentrations were

determined by Qubit™ fluorometer (Invitrogen™). Measurements of the DNA in the samples were undertaken in parallel with known standard solutions.

### 7.3.4.2 Target quantification using qPCR

The quantification of *qnrS* gene was performed through real-time PCR (qPCR) system (StepOnePlus, Applied Biosystems). The following primers were used for specific amplification of *qnrS* gene (Eurofins Genomics, Germany) (Table 7-2).

**Table 7-2** Details of *qnrS*.

| Gene | Primer | Sequence (5' -> 3') |
|---|---|---|
| *qnrS* | qnrSrtF11 | GACGTGCTAACTTGCGTGAT |
| | qnrSrtR11 | TGGCATTGTTGGAAACTTG |

The PCR conditions were programmed with an initial denaturation at 95 °C for 10', followed by 40 cycles at the same temperature for 15 seconds and an annealing temperature of 60 °C for a minute. A melt curve was successively performed starting from 65 °C to end up to 95 °C (Figure S5). qPCR reaction was performed in duplicate in a 25 µL volume mixture and conducted in 96 well plates containing 12.5 µL of SYBR Green Master Mix (Applied Biosystems), 0.1 µM of each primer and 5 µL of template DNA. Amplicon cloning was performed by insertion of the PCR product into a plasmid pCR™ 2.1-TOPO® TA vector (Invitrogen™) in the cloning reaction. The following equation (14) was used for calculating the copy number $\mu L^{-1}$ as described elsewhere [2]:

$$\frac{\text{Copy number}}{\mu\text{L}} = \frac{\text{Plasmid DNA concentration x Avogadro's number}}{\text{Plasmid length x 660}} \quad (14)$$

Where *Plasmid DNA concentration* is expressed in g $\mu L^{-1}$ and *Plasmid length* in bp. 660 is the average molecular weight of 1 bp [7]. Indeed, by ten-fold dilutions of the positive sample, a standard curve was created in order to quantify absolute concentration in European wastewater samples (Figure S6).

#### 7.3.4.3 Target quantification using Digital PCR

Digital PCR analysis was performed on the QuantStudio® 3D Digital PCR System (Life Technologies, Thermo Fisher Scientific). The digital PCR reaction mixture consisted of QuantStudio® 3D Digital PCR Master Mix, 20X SYBR® Green I dye in TE buffer at pH 8, each primer and DNA sample. The mixture was loaded in a high-density nanofluidic chip to partition the sample in many independent reactions and sealed. The thermal cycling program was the same reported for qPCR analysis in the previous paragraph. AnalysisSuite™ software was used to get quantification of the targeted gene and statistical analysis of the results.

## 7.4 Results and Discussion

### 7.4.1 Analysis of antibiotics in wastewater

This study targets an antifungal, quinolones and fluoroquinolones. The latter class is a high priority class among antibiotics according to WHO [8]. Reported by the European Centre for Disease Prevention and Control (ECDC) in 2014 [9], consumption of quinolones for systemic use in the community (primary care and hospital sector), expressed in defined daily dose (DDD) per 1000 inhabitants and per day in presented in Table 7-3.

**Table 7-3** Community consumption, relative consumption and seasonal variation of quinolones in 2014 according to ECDC report (n.a. means not available).

| Country | Community Consumption[a] | Hospital sector[a] | Relative consumption[b] | Seasonal variation | Tot consumption[c,d] |
|---|---|---|---|---|---|
| Norway | 0.50 | 0.07 | 3.2% | - | 0.57 |
| UK | 0.48 | 0.11 | 2.3% | 9.8% | 0.59 |
| Denmark | 0.50 | 0.21 | 3.1% | 4.9% | 0.71 |
| The Netherlands | 0.79 | 0.11 | 7.4% | - | 0.90 |
| Switzerland | n.a. | | n.a. | n.a. | n.a. |
| Italy | 3.41 | 0.41 | 12.2% | 22.0% | 3.82 |
| Spain | 2.31 | - | 10.6% | - | 2.31 |

[a] expressed as DDD per 1000 inhabitants and per day

[b] Consumption of fluoroquinolones expressed as % of the total consumption of antibacterials for systemic use

[c] expressed as the total of consumption from primary care and hospital sector

[d] data not provided by ECDC

As reported by EFSA [8], the underestimation of the fluoroquinolones consumption in humans is less problematic with respect to 3rd- and 4th-generation cephalosporins, especially because fluoroquinolones are mostly used in human medicine. Indeed, according to ECDC/EFSA/EMA first joint report [8], their consumption in 2012 was higher in humans than in food-producing animals for most countries with a population-weighted mean of 7.04 mg/kg estimated biomass and corresponding ranges of 2.24–16.03 mg/kg. Therefore, for the locations involved in that study (although no data for Switzerland were available), it was possible to state that:

- Denmark, Norway and the Netherlands showed similar human consumption range (≤5 mg per kg estimated biomass) with negligible income from food-producing animals.
- For countries where only community consumption data were available for human medicine, such as the UK and Spain, the UK had the lowest population-corrected consumption of fluoroquinolones with a slight contribution from food-producing animals. Spain had high amount population-corrected fluoroquinolones consumption estimates for human use (>10 mg per kg estimated biomass) and for food-producing animals (< 5 mg per kg estimated biomass).
- Italy had the highest difference between population-corrected fluoroquinolones consumption estimates for human use (~15 mg per kg estimated biomass) and food-producing animals (<5 mg per kg estimated biomass).

Results on population-mass loads for the studied analytes are shown in Figures 7-3, 7-5 and 7-6. Table S1 provides concentrantions, daily population-normalised mass loads (and EF values for chiral compounds) for every day of the week-monitoring campaign.

#### 7.4.1.1 Ciprofloxacin

Ciprofloxacin is an achiral synthetic drug. In humans 40-50% of ciprofloxacin is excreted unchanged and as desethylene-ciprofloxacin and sulfo-ciprofloxacin with antibacterial activity 30 times less than ciprofloxacin at 2% and 4%, respectively, oxo-ciprofloxacin with an activity 10-times lower at 7% and sporadically as formylciprofloxacin (a minor metabolite) (Figure 7-4) [10, 11].

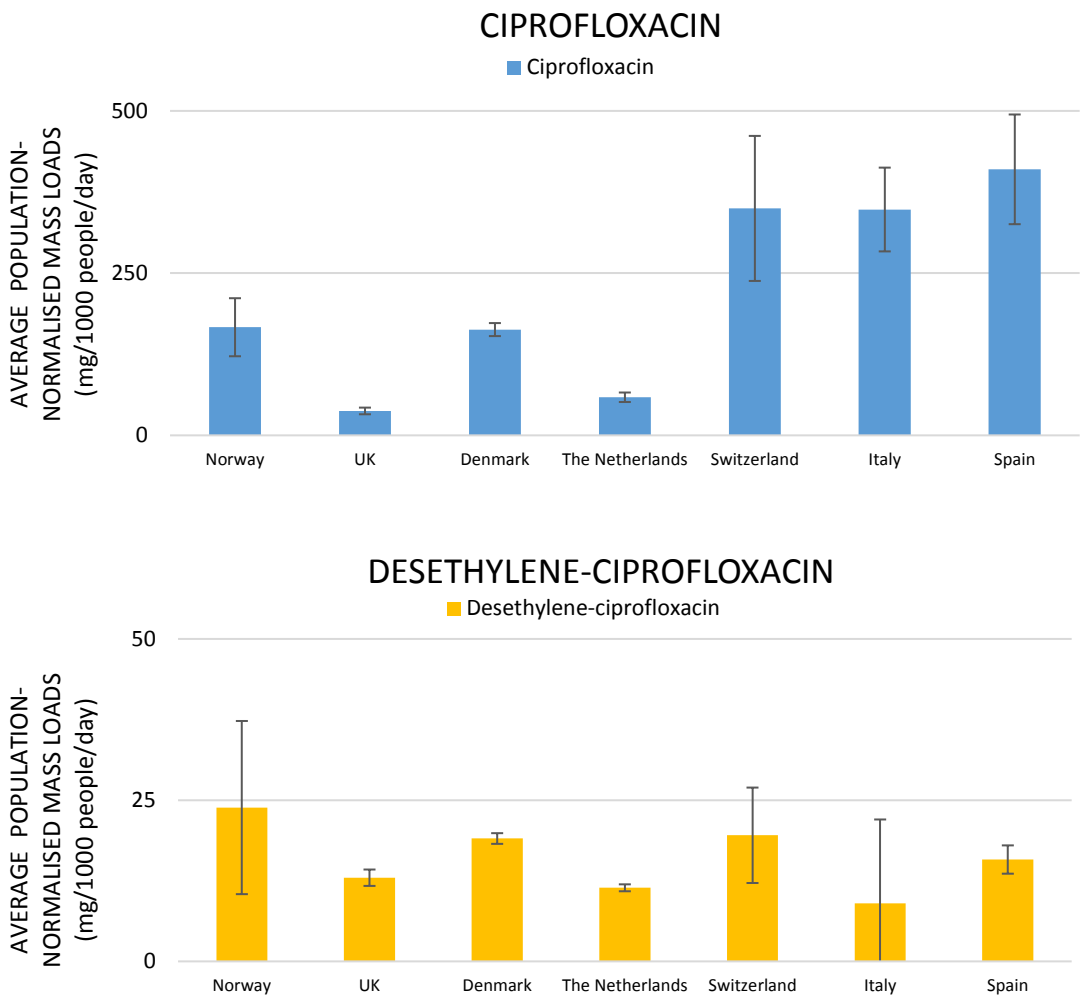



**Figure 7-3** Average population-normalised mass loads for ciprofloxacin and its metabolite. Here, results are shown with the fullnames of the countries (and not with those of the cities).

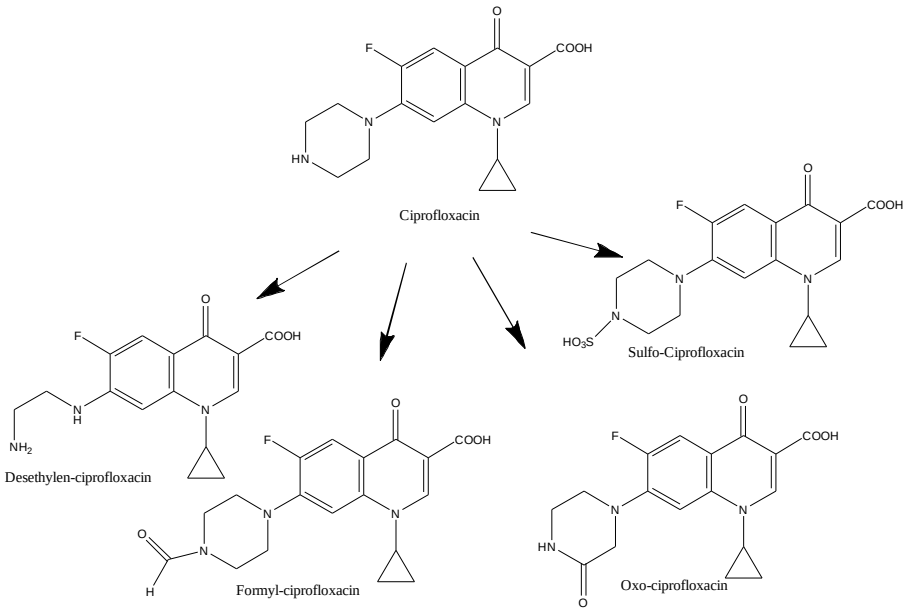



**Figure 7-4** Metabolism of ciprofloxacin.

Ciprofloxacin is also a metabolite of enrofloxacin, which is a veterinary drug. Metabolism of enrofloxacin leads to excretion of 31% as ciprofloxacin, 5% as oxo-ciprofloxacin and 3% as desethylene-ciprofloxacin [12]. Ciprofloxacin and its metabolite desethylene-ciprofloxacin were selected as biomarkers of ciprofloxacin use.

In this study, population-normalised ciprofloxacin loads ranged from a minimum average value of 37.6 mg $day^{-1}$ 1000 $people^{-1}$ in Bristol to a maximum value of 409.9 mg $day^{-1}$ 1000 $people^{-1}$ in Castellon. The metabolite loads varied from 9 mg $day^{-1}$ 1000 $people^{-1}$ in Milan to a maximum value of 23.9 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo. The highest intra-week variability was found for ciprofloxacin in Zurich, followed by Castellon and Milan, for desethylene-ciprofloxacin in Oslo and Milan. Indeed, most of the countries showed stable mass loads over the week (Table S1).

Intra-day variation was observed by Coutu et al. (2013) [13], where a peak was found between 7 and 9 a.m. due to accumulation of the excreted drug in urine during night-time and posology which caused the increased loads at the first flush in wastewater [14]. Because of its therapeutic use, seasonal variations were also found by Coutu et al. (2013) in Switzerland [13]. This seasonal trend is most likely in central and southern European countries, rather than Northern countries, where a drop in use during summer is observed, especially because of high temperatures.

The ratio between parent compound:metabolite ranged between 3:1 and 8:1 for Northern European cities and around 20:1 for Southern ones. According to metabolism data, the ratio indicating human consumption is nearly 22.5:1, thus the loads of ciprofloxacin from Southern cities may be mainly related to consumption.

From PCA data available for the UK in March 2015 [15], 510 kg of ciprofloxacin were prescribed. Considering 45% as average excretion percentage for the parent compound and 2% for the metabolite, the excreted amount was calculated as 229.6 and 10.2 kg respectively. According to the available statistics, its consumption was estimated at 210 mg $day^{-1}$ 1000 $people^{-1}$ (Table 7-4). Using an average dose of 400 mg from the oral formulations available, the estimates found from wastewater analysis were 94.2 and 793.9 mg $day^{-1}$ 1000 $people^{-1}$ using ciprofloxacin and desethylene-ciprofloxacin respectively as DTRs.

**Table 7-4** Consumption estimates were calculated considering prescriptions data from PCA [15] (England only) and wastewater (WW) analysis. Sorption was not considered in the estimates.

| **Pharmaceuticals** | **Total consumption (kg/month)** | **DTR** | **Consumption estimates (mg day$^{-1}$ 1000 people$^{-1}$)** | |
|---|---|---|---|---|
| | | | NHS data (2015) | WW analysis (2015) |
| Ciprofloxacin | 510.3 | Ciprofloxacin | 210.5 | 94.2 |
| | | Desethylene-ciprofloxacin | | 793.9 |
| Ofloxacin | 18.6 | Ofloxacin | 4.2 | 17.2 |
| | | Ofloxacin-*N*-oxide | | 45.9 |
| | | Desmethyl-ofloxacin | | 141.8 |
| Norfloxacin | 0.1 | Norfloxacin | 0.1 | 2.9 |
| Nalidixic acid | 0.0 | Nalidixic acid | 0.3 | 0.9 |
| Lomefloxacin | - | Lomefloxacin | - | 0.8 |
| Moxifloxacin | 3.2 | Moxifloxacin | 3.0 | - |
| | | Moxifloxacin-*N*-sulphate | | - |

This underestimation in wastewater calculations may be explained because of the high adsorption to the particulate phase of wastewater for ciprofloxacin that reaches 68% according to Petrie et al. 2014 [15]. Indeed, this aspect was also highlighted by Petrie et al. 2015 [16]. If the absorption to the suspended particulate matter is re-considered in loads, estimates in wastewater are 294.5 mg day$^{-1}$ 1000 people$^{-1}$ using ciprofloxacin as DTR. This data seems more in agreement with the official statistics.

#### 7.4.1.2 Ofloxacin

After (±)-ofloxacin intake, ofloxacin urinary metabolites are (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin with a percentage of excretion of 2%, (±)-ofloxacin-glucuronide along with the parent drug itself at 80-85%.

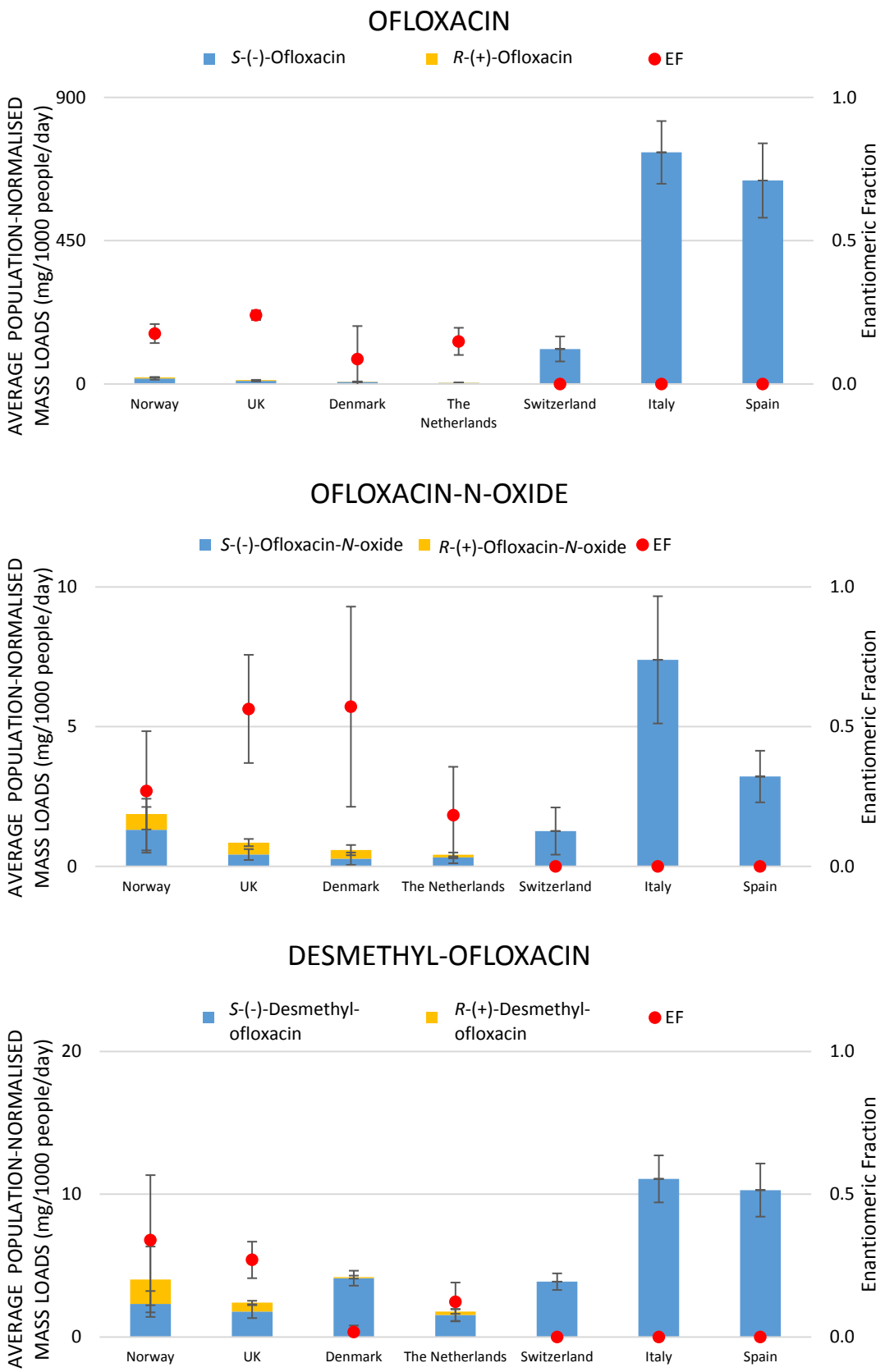



**Figure 7-5** Average population-normalised mass loads for ofloxacin and its metabolites. Mean EFs were shown in the secondary vertical axis. Results are shown with the fullnames of the countries (and not with those of the cities).

Disposition of ofloxacin was found to be stereoselective in humans probably due to differences in renal excretion [17]. Stereoselective intake of the *S*-(-)-ofloxacin (well known as *levo*-ofloxacin) is linked to the production of *S*-(-)-form metabolites.

The selection of ofloxacin biomarkers in wastewater was based on (±)-ofloxacin, (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin. This was the first time that a complete investigation of ofloxacin metabolic profile was performed in wastewaters in a pan-European study.

In the current study, population-normalised ofloxacin loads ranged from a minimum average value of 4.3 mg $day^{-1}$ 1000 $people^{-1}$ in Utrecht to a maximum value of 727.4 mg $day^{-1}$ 1000 $people^{-1}$ in Milan. The same was found for the metabolites, but with lower mass loads due to their low urinary excretion. In fact, they ranged between 0.4 and 7.4 mg $day^{-1}$ 1000 $people^{-1}$ for ofloxacin-*N*-oxide and between 1.8 and 11.8 mg $day^{-1}$ 1000 $people^{-1}$ for desmethyl-ofloxacin. The highest intra-week variability was found for ofloxacin in Milan and Castellon, whilst quite stable mass loads were achieved for both metabolites (Table S1).

The most important considerations came out from the analysis of enantiomeric profiling. A predominance of the *S*-(-)-ofloxacin loads was observed with respect to *R*-(+)-ofloxacin in northern European cities. The contribution of only one enantiomer, *S*-(-)-form, was exclusively found in southern locations, thus showing a stereoselective usage of the drug (probably linked with prescriptions of *S*-(-)-enantiomer).

The investigation of ratios were performed through calculations of their mass loads. A constant ratio 3:1 for the two ofloxacin enantiomers was found over the week in Bristol, whist 4:1 in Oslo and Utrecht. Ofloxacin:ofloxacin-*N*-oxide ratio was 10:1 for northern cities, whilst it was different for the others. Ofloxacin was nearly six times higher than desmethyl-ofloxacin in Oslo and Bristol, whilst it was three times higher in Lyngby and Utrecht. According to metabolism data, the proposed ratio was 41.2:1. In Castellon and Milan, slightly higher ratios were found, thus suggesting also disposal of ofloxacin in these cities.

According to PCA data in March 2015 from the UK [15], 18.6 kg of ofloxacin were prescribed. Considering 82.5% as average excretion percentage for

the parent compound and 2% for the metabolites, the excreted amount was calculated as 15.3 and 0.4 kg respectively. Therefore, on the basis of the available statistics, its consumption was estimated at 4.2 mg $day^{-1}$ 1000 $people^{-1}$ (Table 7-4). Using an average dose of 300 mg, the estimates found from wastewater analysis were 17.2, 45.9 and 141.8 mg $day^{-1}$ 1000 $people^{-1}$ using ofloxacin, ofloxacin-*N*-oxide and desmethyl-ofloxacin respectively as DTRs. These estimates through wastewater analysis do not take into account the influence of the adsorption to the suspended particulate matter (63% according to Petrie et al. 2014 [15]). If this parameter is considered in the calculations, estimates in wastewater are 46.4 mg $day^{-1}$ 1000 $people^{-1}$ considering ofloxacin as DTR. Despite that, official and wastewater analysis did not provide similar estimates.

#### 7.4.1.3 Norfloxacin

Norfloxacin is an achiral synthetic fluoroquinolone. From 25 to 40% of the dose it is excreted as unchaged in urine (30% as average in faeces within 48 hours) and as metabolites at 5-10% within 24-48 hours.

In wastewater, population-normalised loads were <MDL in Lyngby and in Utrecht up to a maximum value of 40.2 mg $day^{-1}$ 1000 $people^{-1}$ in Zurich. As in the case of ciprofloxacin and ofloxacin, intra-day variation was observed for norfloxacin by Coutu et al. (2013) with a peak load in wastewater at the first flush in early morning [13].

From the UK, PCA data shows that 0.1 kg were dispensed in March 2015 [15]. Considering 32.5% as average excretion percentage, the excreted amount calculated was 0.04 kg. Hence, its consumption was estimated at 0.1 mg $day^{-1}$ 1000 $people^{-1}$ (Table 7-4). Estimates from wastewater analysis were 2.9 mg $day^{-1}$ 1000 $people^{-1}$ showing a slight disagreement between two sets of data.

#### 7.4.1.4 Nalidixic acid

Nalidixic acid is an achiral synthetic quinolone. In humans, only ~2-3% of nalidixic acid is excreted unchanged, 80% is metabolised to 7-hydroxy-nalidixic acid, which is an active compound, carboxy metabolite, the inactive conjugates (7-hydroxy-nalidixic acid and nalidixic acid glucuronides).

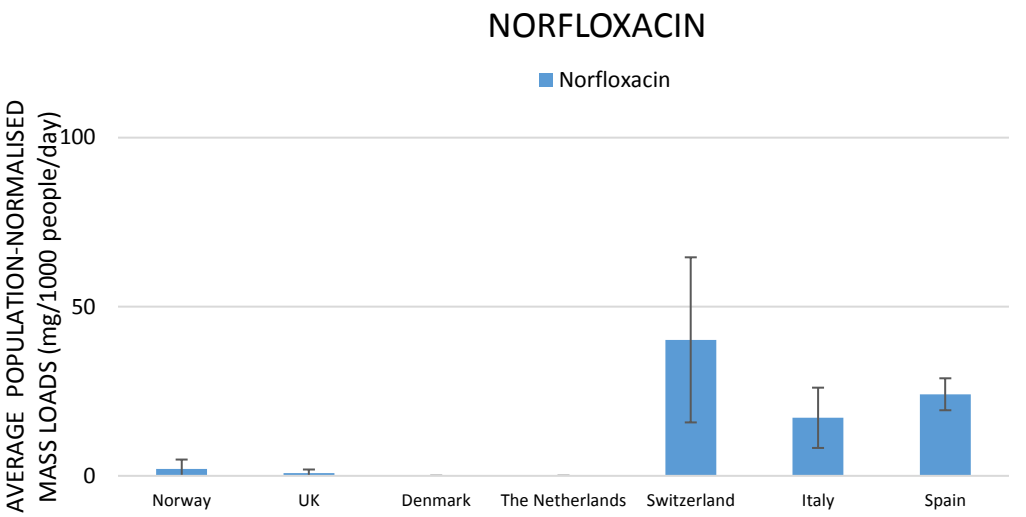
In this study, population-normalised loads were <MDL in Lyngby up to a maximum value of 2.5 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo. PCA data from the UK showed that this drug was dispensed in the amount of 0.036 kg only by pharmacies and appliance contractors in England. Considering 2% of excretion, the excreted amount calculated was very low (0.001 kg). Thus, its consumption was estimated at 0.3 mg $day^{-1}$ 1000 $people^{-1}$. The possible hydrolysis of the glucuronides and, thus, the release of free-nalidixic acid can contribute to loads found in wastewater. As the excretion of these glucuronides is not an available data, estimates were performed considering a total contribution of 40% of the parent compound. Estimates from wastewater analysis were 0.9 mg $day^{-1}$ 1000 $people^{-1}$ using nalidixic acid as DTR (Table 7-4). Even if both estimates agreed quite well, other DTRs could be still investigated.

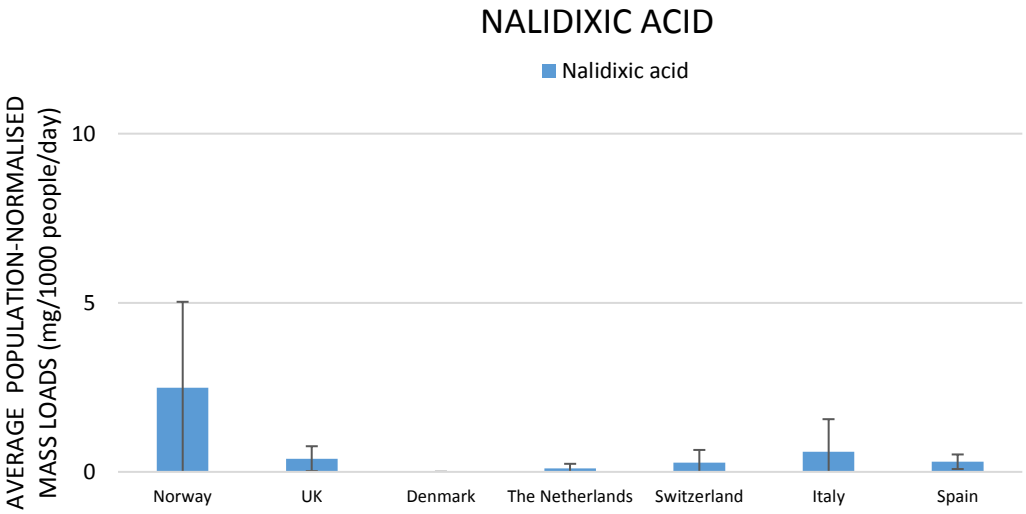
#### 7.4.1.5 Lomefloxacin

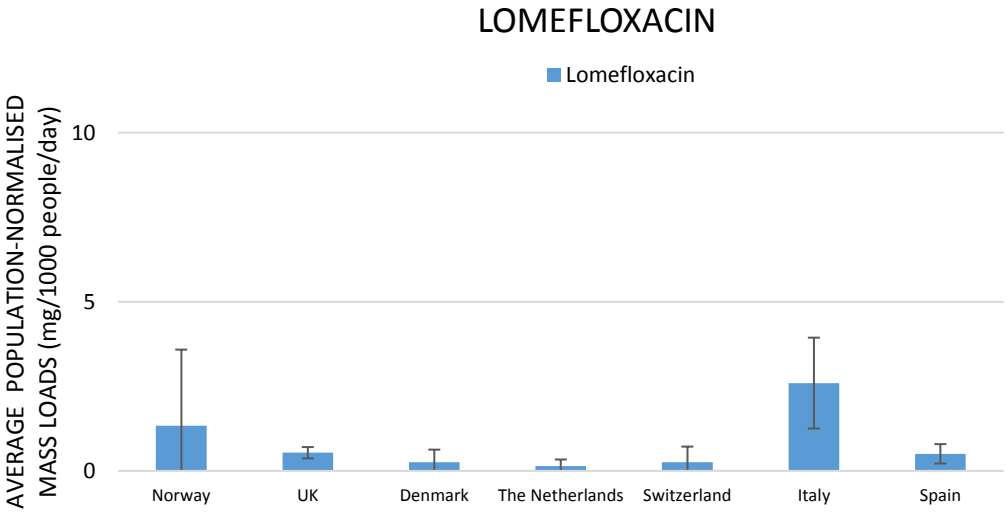
(±)-Lomefloxacin is a chiral synthetic fluoroquinolone. Once ingested, 65% is found unchanged in the urine and 9% is excreted as glucuronide. To the author's knowledge, information on stereoselective metabolism is not available. Unfortunately, under chromatographic conditions used, its enantiomers are not resolved. Therefore, analyses of its loads are intended for (±)-lomefloxacin.

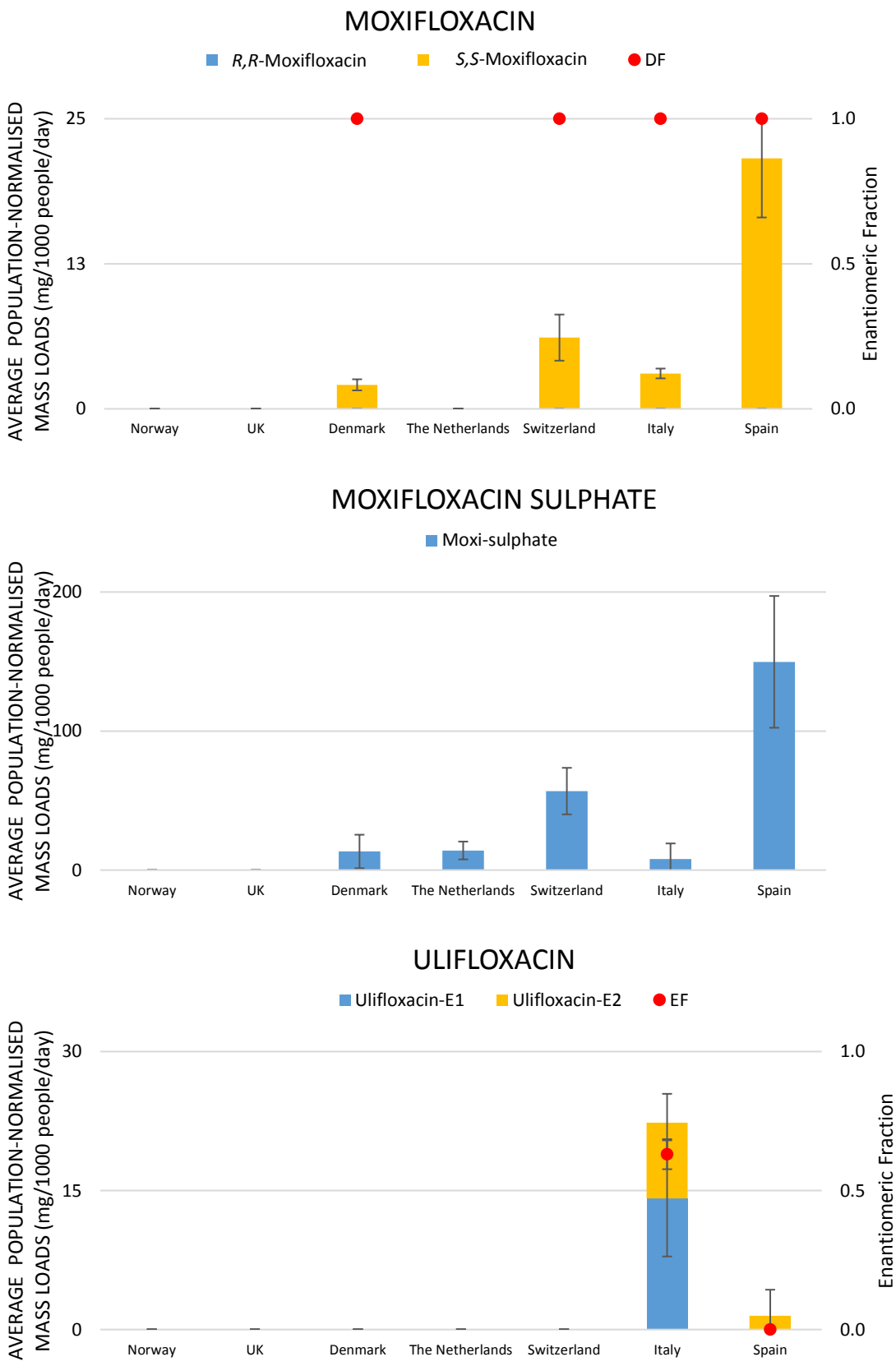
In this study, population-normalised loads ranged from a minimum value of 0.1 for Utrecht to a maximum value of 2.6 mg $day^{-1}$ 1000 $people^{-1}$ for Milan.

Even if (±)-lomefloxacin was not dispensed in England in March 2015 according to PCA data, population-normalised loads were found in wastewater at 0.5 mg $day^{-1}$ 1000 $people^{-1}$ (Table 7-4). Its estimate takes into account also the percentage fraction from the glucuronides (assuming that they are hydrolysed in wastewater). Considering 74% as the excretion percentage, the consumption was estimated at 0.8 mg $day^{-1}$ 1000 $people^{-1}$.


NORFLOXACIN
Norfloxacin
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
100
50
0
Norway
UK
Denmark
The Netherlands
Switzerland
Italy
Spain


NALIDIXIC ACID
Nalidixic acid
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
10
5
0
Norway
UK
Denmark
The Netherlands
Switzerland
Italy
Spain


LOMEFLOXACIN
Lomefloxacin
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
10
5
0
Norway
UK
Denmark
The Netherlands
Switzerland
Italy
Spain


MOXIFLOXACIN
R,R-Moxifloxacin
S,S-Moxifloxacin
DF
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
Enantiomeric Fraction
25
13
0
1.0
0.5
0.0
Norway
UK
Denmark
The Netherlands
Switzerland
Italy
Spain
MOXIFLOXACIN SULPHATE
Moxi-sulphate
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
200
100
0
Norway
UK
Denmark
The Netherlands
Switzerland
Italy
Spain
ULIFLOXACIN
Ulifloxacin-E1
Ulifloxacin-E2
EF
AVERAGE POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)
Enantiomeric Fraction
30
15
0
1.0
0.5
0.0
Norway
UK
Denmark
The Netherlands
Switzerland
Italy
Spain

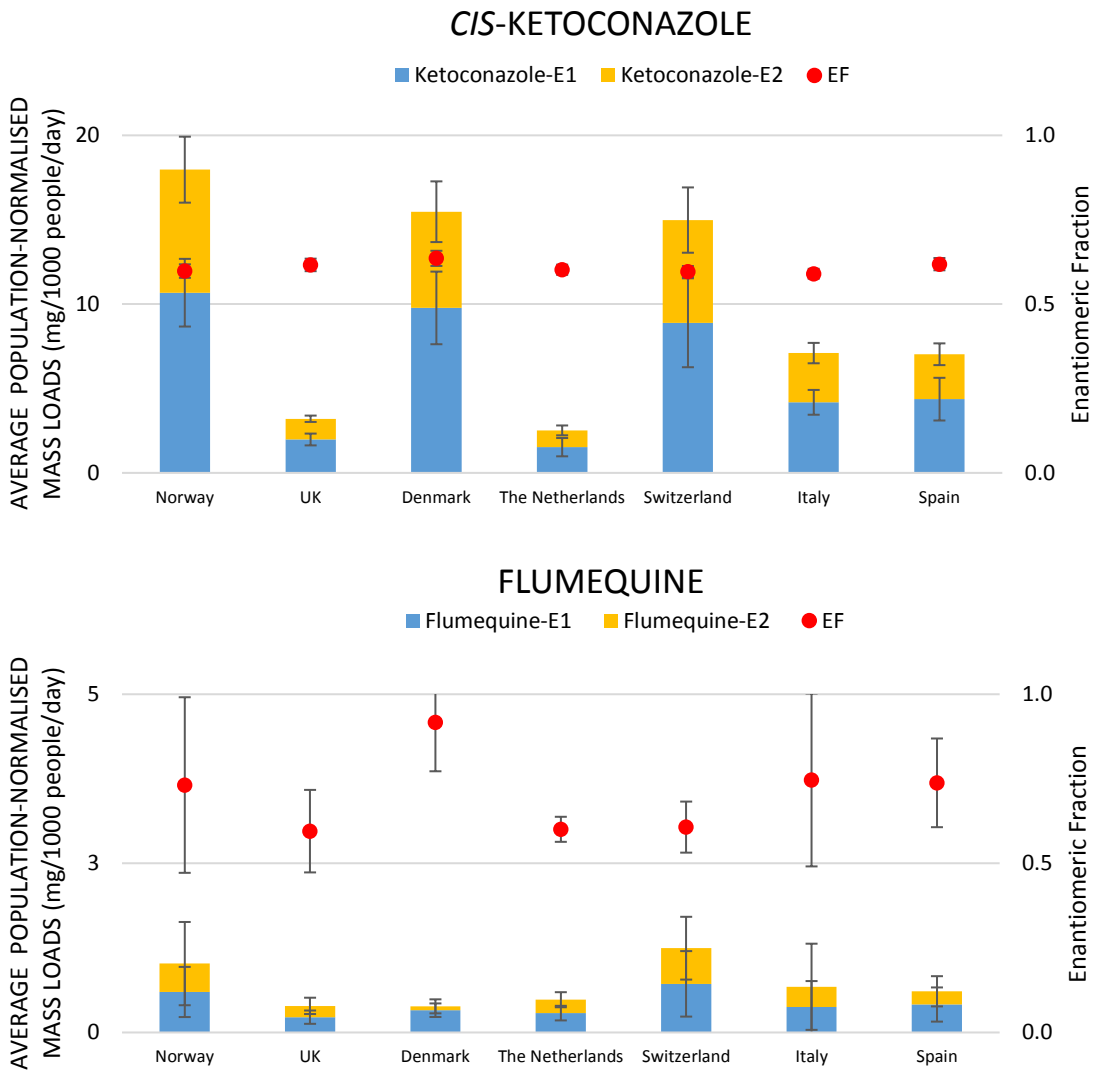



**Figure 7-6** Average population-normalised mass loads for other quinolones investigated and *cis*-ketoconazole. For chiral drugs, which enantiomers were separated under chromatographic conditions used, mean EF was also shown on the secondary vertical axis. Results are shown with the fullnames of the countries (and not with those of the cities).

### 7.4.1.6 Moxifloxacin

(±)-Moxifloxacin is a synthetic fluoroquinolone that has two chiral centres. It is sold in one form of *S,S*-moxifloxacin. *R,R*-moxifloxacin is an impurity of the drug, therefore it is unlikely a product of human metabolism. Indeed, *S,S*-moxifloxacin is excreted unchanged at about 20% in urine and 25% in faeces, as acyl-glucuronide at 14% of the dose in urine and moxifloxacin-*N*-sulphate at 35% of the dose in faeces. In this study, diastereomers of moxifloxacin were separated under selected chromatographic conditions. Hence, it was possible to verify whether *R,R*-moxifloxacin was present in the environmental matrix due to possible

microbial conversion of the parent drug. *S,S*-moxifloxacin and moxifloxacin-*N*-sulphate were selected as biomarkers of moxifloxacin human use.

Here, population-normalised moxifloxacin loads ranged from <MDL to a maximum of 21.6 mg $day^{-1}$ 1000 $people^{-1}$ in Castellon enriched of only one S,S-enantiomer. Population-normalised moxifloxacin-*N*-sulphate loads ranged from zero to a maximum of 149.8 mg $day^{-1}$ 1000 $people^{-1}$ in Castellon enriched of only one *S,S*-enantiomer as the parent compound.

Even if (±)-moxifloxacin was not found in wastewater samples from Bristol, PCA data from March 2015 [15] shows that *S,S*-moxifloxacin was dispensed in the amount of 3.2 kg. Therefore, estimates of its consumption was 3.0 mg $day^{-1}$ 1000 $people^{-1}$.

#### 7.4.1.7 Prulifloxacin

(±)-Prulifloxacin is a synthetic prodrug sold as racemate for oral administration. It is converted in its active compound, ulifloxacin, by hepatic enzyme. The chiral centre is not the metabolic site and there is no stereoselective metabolism. Only *L*-ulifloxacin has the bactericidal effects, but still enantiomerically pure *L*-form is not commercially available yet [18]. Ulifloxacin is excreted at 17-23% in the urine and 17-29% in the faeces.

In the current study, population-normalised ulifloxacin loads were found in Milan and Castellon with values at 22.3 and 1.5 mg $day^{-1}$ 1000 $people^{-1}$ respectively. Enrichment of ulifloxacin first-eluting enantiomer was detected through chiral analysis in Milan, whilst the opposite was observed in Castellon. (±)-Prulifloxacin was expected to be found in the wastewaters only in case of direct disposal. However, it was not found in any sample, which indicates that there was no direct disposal of this drug.

#### 7.4.1.8 Ketoconazole

(±)-Ketoconazole is a synthetic antifungal, sold as a racemate of the *cis*-configuration, i.e. (+)-ketoconazole and (−)-ketoconazole. It is excreted in a percentage of nearly 13% in urine (of which 2-4% as unchanged) [19].

In this study, population-normalised loads were from 2.5 in Utrecht up to 18 mg $day^{-1}$ 1000 $people^{-1}$ in Oslo. (±)-*cis*-Ketoconazole was enriched with the first-eluting enantiomer, thus suggesting a potential enantioselective metabolism. This may be controversial, as Hamdy and Brocks [20] found evidence of non-linear stereoselective pharmacokinetics in rats and no stereoselective metabolism by liver microsomes. Therefore, further works are needed to support this finding, especially in-sewer stability studies for proving no selective enantio-biodegradation.

According to ECDC in 2014, the consumption of ketoconazole for systemic use in the community (primary care and hospital sector), expressed as defined daily dose (DDD) per 1000 inhabitants and per day, is reported in Table 7-5 for the countries of the selected locations.

Hence, the loads found in wastewater are more linked to other sources of ketoconazole (e.g. topical absorption from anti-infective skin preparations) or to the veterinary loads.

**Table 7-5** Consumption and relative consumption of ketoconazole in 2014 according to ECDC (n.a. means not available):

| **Country** | **Community Consumption[a]** | **Hospital sector[a]** |
|---|---|---|
| Norway | 0.00 | <0.01 |
| UK | n.a. | n.a. |
| Denmark | 0.00 | 0.00 |
| The Netherlands | <0.01 | - |
| Switzerland | n.a. | n.a. |
| Italy | 0.00 | 0.00 |
| Spain | n.a. | n.a. |

[a] expressed as DDD per 1000 inhabitants and per day

#### 7.4.1.9 Flumequine

(±)-Flumequine is a racemic drug marketed in the veterinary market. After enzyme deconjugation, it is excreted as unchanged 81-86% in calves urine, at 12-17% as 7-hydroxy-flumequine in calves urine and as glucuronide conjugates. It undergoes stereoselective metabolism in sheep, cattle and poultry.

Population-normalised loads ranged from 0.4 in Lyngby to 1.2 mg $day^{-1}$ 1000 $people^{-1}$ in Zurich. Enrichment of flumequine first-eluting enantiomer was detected in all the samples from the locations investigated.

Its presence in this environmental matrix can be explained as excretion product from animals' metabolism (EF>0.5) or as its direct disposal (EF=0.5). In wastewater, EF values ranged from 0.6 ± 0.0 to 0.9 ± 0.1). In this case, the first option seems to be the most plausible because of the chiral signature (EF>0.5) in all the samples.

#### 7.4.1.10 Other quinolones

Racemic (±)-nadifloxacin is the active compound in some topical anti-infective creams. The isomer *S*-(-)-nadifloxacin has higher antibacterial activity with respect to the other enantiomer. Its metabolism produces less than 5% of unchanged nadifloxacin excreted in urine and conjugates, such as sulphate and glucuronides, at about 20% [21]. In wastewater, glucuronides can be potentially re-converted to the parent compound after hydrolysis by faecal bacteria.

*R*-(+)-besifloxacin is a synthetic quinolone formulated for ophthalmic use. It is sold only in one enantiomeric form. After human hepatocytes incubation, it does not go through chiral interconversion to its enantiomer [22].

As both were not detected at enantiomeric level in composite wastewater samples, their enantiomeric profiling could not be investigated.

### 7.4.2 Qualitative test in selective media

Wastewater samples were incubated in CLED agar, which is a differential culture medium for isolating bacteria from the suspected cases of urinary tract infection in urine specimens [23]. From the typical colony morphology on CLED agar it was possible to observe several bacteria, such as *Escherichia coli* with its opaque yellow colonies with a deeper yellow centre, *Klebsiella*, mucoid yellow and whitish-blue colonies, *Enterococci*, characterised by small yellow colonies and *Staphylococcus aureus* with its deep and uniform yellow colonies and *Coagulase Negative Staphylocci* with pale opaque yellow colonies. Results are summarised in Table S2.

### 7.4.3 Target *qnr* gene quantification

*QnrS* gene was selected because of its reduced susceptibility to fluoroquinolones according to Rodriguez-Mozaz et al. [2] and Marti et al. [25]. Target *qnrS* quantification was performed using two techniques: qPCR and digital PCR.

Standard curve, created through qPCR analysis, was linear in the studied range. The PCR efficiencies ranged from 90% to 105%. A good separation between amplified and non-amplified reactions was achieved with digital PCR. The advantage in using digital PCR was the possibility to quantify the targeted gene in the wastewater DNA sample without the use of a standard curve along with the high number of independent reactions performed per chip. Results from qPCR analysis were expressed as Ct (threshold cycle) values, which are relative measurements of the concentration of the target gene. By using the equation from the standard curve, they were then quantified as "log *qnrS* copies $\mu L^{-1}$". Results from digital PCR analysis were given directly as copies $\mu L^{-1}$ (Figure 7-7). Both sets of results were converted in millilitres unit. In order to remove any variation in flow, daily loads were calculated starting from "absolute concentration" (this was because results were not normalised with *16S rRNA*). A comparison of the results with two techniques is shown in Figure 7-8.

A higher absolute copy number of *qnrS* gene was observed for Italy, the UK and Norway by using qPCR, whilst they were in the reversed order according to digital PCR. Levels of *qnrS* gene were negligible for Switzerland through qPCR and nearly close to levels detected in Spain through digital PCR.

### 7.4.4 Analysis of quinolones and *qnrS* gene loads in wastewater

In this study, most of the compounds belong to the fluoroquinolones class. As *qnrS* is not specific for any quinolone, a calculation based on the total of the concentrations and the population-normalised loads of all studied quinolones/fluoroquinolones was performed in order to compare the results with findings from PCR data (Figure 7-9). The highest occurrence was found in Italian and Spanish samples.

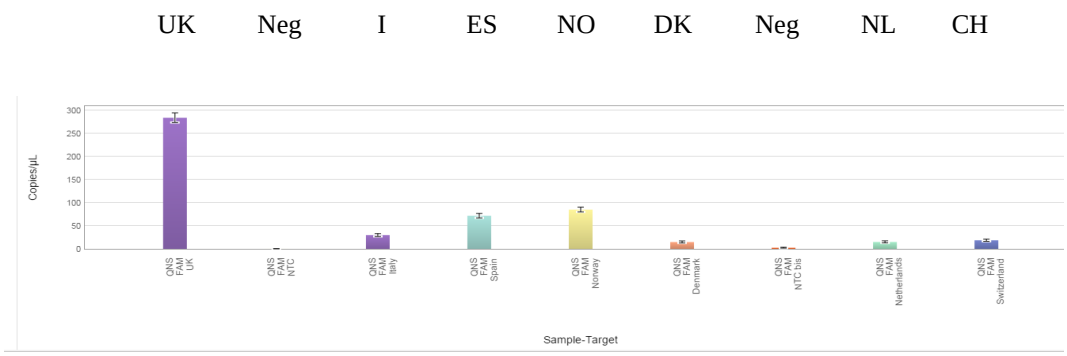



**Figure 7-7** "Concentration" of *qnrS* gene through digital PCR in the European wastewater samples. These samples were collected on the same day in all the sampling sites across Europe (Two negative controls were tested, *Neg*.). Results are shown with the acronyms of the countries (and not with the fullnames of the cities).

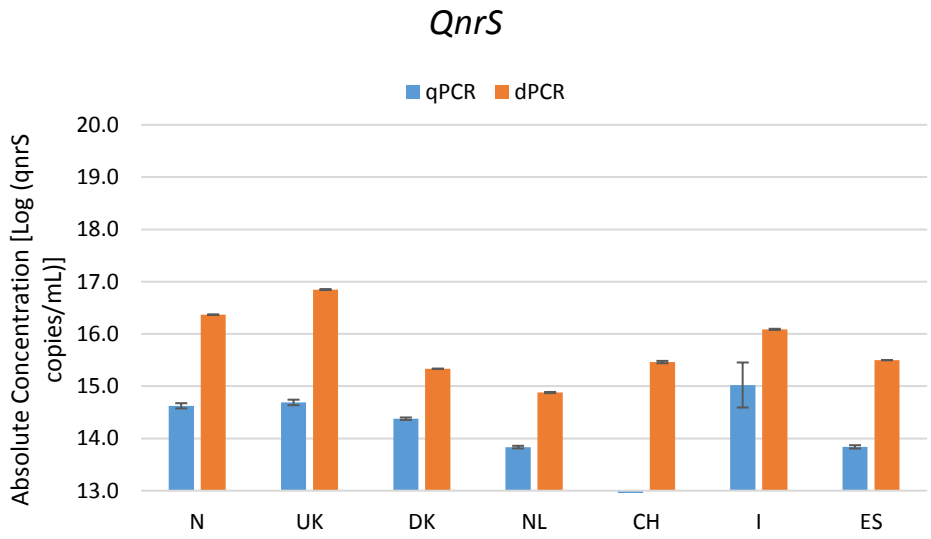



**Figure 7-8** "Absolute concentration" of *qnrS* gene obtained with qPCR and digital PCR in the European wastewater samples. These were calculated as gene loads. These samples were collected on the same day in all the sampling sites across Europe. Results are shown with the acronyms of the countries (and not with the fullnames of the cities).

This data was in accordance with the report by ECDC from 2014 (Table 7-3) [9]. A higher amount was expected to be found in Spain from PCR data. Moreover, it is also true that the population size of the Spanish city considered in the study was the lowest when compared to other European locations (population ≥300000). This could mean that population normalised gene loads need to be considered. Moreover, a higher absolute copy number in the UK and Norway could be explained by colder weather conditions that may have contributed to preserve a higher *qnrS* copy number.

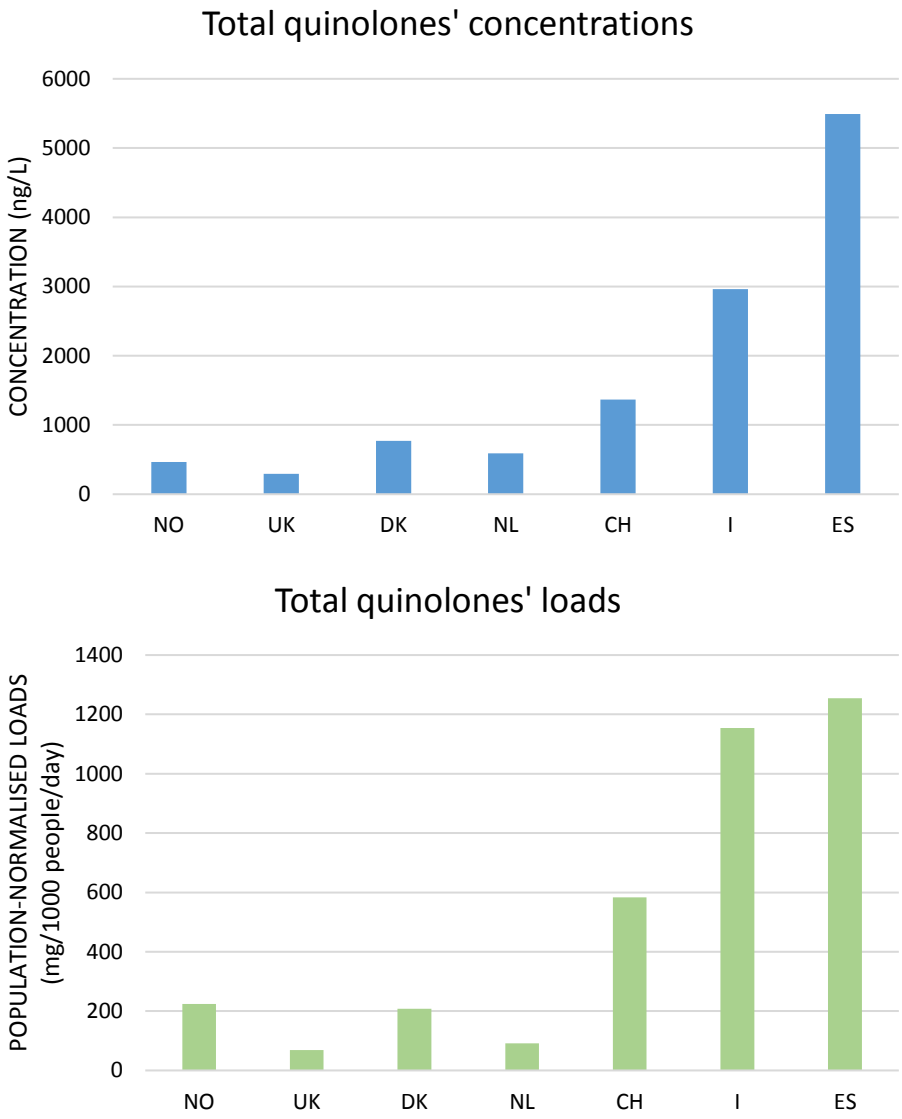



**Figure 7-9** Average concentrations and average population-normalised mass loads calculated from quinolones'data in one city for each country. For simplicity acronyms of the countries are used in the graphics, instead of the fullnames of cities.

A limitation of both analyses was represented by missing relative quantification with 16S rRNA gene copy number, which could have helped in the normalisation of the data. Thus, this is highly recommended in future studies. Antimicrobial resistance could be enhanced by the spreading of antibiotics level in the environment especially when direct disposal of some pharmaceuticals occurred.

ECDC/EFSA/EMA first joint report worked on the relationship between national consumption of fluoroquinolones/quinolones and the risk of reduced susceptibility to ciprofloxacin by using *E. coli*, *Salmonella spp.*, *C. coli* and *C. jejuni* as indicators

[8]. According to that, the impact on the contribution of quinolone consumption could be explained as follows:

(i) cross-resistance between quinolones and fluoroquinolones are similarly detected by the use of epidemiological cut-off values for ciprofloxacin resistance;

(ii) ciprofloxacin resistance in *E. coli* is leaded by the selection of quinolones for the first mutation step;

(iii) the dissemination of plasmid-mediated resistance to quinolones mediated by *qnr* genes in *Salmonella spp.* can provide opportunities for co-selection of unrelated antimicrobials.

On the other hand, it was also known that differences in the occurrence of ciprofloxacin resistance were presented in countries with similar low level of ciprofloxacin consumption from ciprofloxacin resistance in *C. coli* data. The reasons were ascribed to differences in the fluoroquinolones consumption in years previous to this study and in bacteria resistance spreading among countries [8]. For this reasons, the fact that *qnrS* gene was not so high in terms of copy number in the analysed samples from Italy and Spain with respect to other northern European cities could be probably a matter of time. Despite these findings, a statistical approach that enables a systematic investigation on the correlation analysis between occurrence of antibiotics and occurrence of their gene resistance is recommended. This is also because a demonstration of significant correlation was proved at local level by Rodriguez-Mozaz et al. (2015) [2].

## 7.5 Conclusions

In this study, WBE was a powerful tool that enabled the monitoring of biomarkers for quinolones, fluoroquinolones and an antifungal drug consumption over a week. This monitoring activity was relevant as it allowed to obtain real-time information on quinolones' misuse throughout several European locations. Indeed, this class of compounds was targeted as its large use could be associated to a spreading of antibacterial resistance. The following biomarkers of exposure to antibiotics were studied in this first pan-European study: (±)-ofloxacin with its main metabolites (±)-ofloxacin-*N*-oxide and (±)-desmethyl-ofloxacin, (±)-moxifloxacin, the precursor (±)-prulifloxacin with its active compound (±)-ulifloxacin, (±)-*cis*-

ketoconazole (the only antifungal included), (±)-flumequine, (±)-nadifloxacin and *R*-(+)-besifloxacin. The investigation on their enantiomeric profiling enabled to understand patterns of drug use and spatial drug use estimates in near-real time. Indeed, through the investigation of the occurrence of ciprofloxacin and ofloxacin metabolites, it was possible to get a urinary excretion profiling reflected in the wastewater. Through the calculation of parent:metabolite ratio, it was possible to hypothesise that ofloxacin may be disposed in Southern European cities due to exceeding parent:metabolite ratio with respect to the proposed metabolism ratio. Enantiomeric profiling enabled to distinguish drug residue origin. In fact, an exclusive stereoselective use of *S*-(-)-ofloxacin was observed in Southern cities, whilst racemic ofloxacin was more predominant in Northern European ones (probably due to a difference in prescriptions of the drug itself). Moxifloxacin human intake was demonstrated by the presence of *S,S*-moxifloxacin and *S,S*-moxifloxacin-*N*-sulphate. Enantiomeric profiling of prulifloxacin showed that only its metabolite, ulifloxacin, was found in Milan and Castellon. Therefore, the presence of ulifloxacin was related to prulifloxacin metabolism and disposal of prulifloxacin did not occur in none of the locations monitored. Potential enantioselective metabolism was hypothesised for (±)-*cis*-Ketoconazole as it was enriched with the first-eluting enantiomer. Even if flumequine metabolites were not included, the enrichment of its first-eluting enantiomer in all the samples was mainly caused by the animals' metabolism rather than its direct disposal. However, the occurrence of quinolones in wastewater reflected the spatial trend from estimated quinolones consumption reported by ECDC in 2014 [9].

Moreover, the occurrence of fluoroquinolone resistance gene was investigated in wastewaters from the same European locations. The approach in using simultaneous analytical techniques, such as chiral chromatography and mass spectrometry for the detection of quinolones biomarkers in wastewater, and bioanalytical techniques, such as qPCR and digital PCR for the detection of biomarkers of effect for specific health-related biomarkers, represented a promising means for understanding correlations of their occurrence in the monitored areas. Furthermore, by this study, it was possible to see the potential in applying the same methodologic approach to other classes of antibiotics, which through their

monitoring can take advantage of a better understanding of the antimicrobial resistance spreading.

## 7.6 Contribution

Erika Castrignanò and Barbara Kasprzyk-Hordern planned and designed the study. Richard Bade, Lubertus Bijlsma, J. A. Baz-Lomba, Sara Castiglioni, Erika Castrignanò, Ana Causanilles, Emma Gracia-Lor, Barbara Kasprzyk-Hordern, Ann-Kathrin McCall, Christoph Ort, Benedek G Plósz, Pedram Ramin, Nikolaos I Rousis, Yeonsuk Ryu, Kevin V Thomas, Pim de Voogt, Ettore Zuccato and Felix Hernandez organised the collection of the wastewater samples from their local wastewater treatment plant. Erika Castrignanò analysed the samples and interpreted the results with contribution from Barbara Kasprzyk-Hordern and Ed Feil.

## 7.7 Supplementary Data

The following supplementary data are contained in Appendix 5:

Table S1 Concentrations (Conc.), daily population-normalised mass loads (Loads) and EF values for chiral compounds for the European monitoring campaign across Europe (besifloxacin, *R,R*-moxifloxacin, prulifloxacin, nadifloxacin were not reported as all values were <MDL). M., T., W., T., F., S., S. were the initials used for indicating the days of the week.

Figure S1 Test "dry run on non-selective media" performed by using 100 µL (on the left) and 200 µL (on the right) of refrigerated wastewater Bristolian samples for day $6^{th}$ and $7^{th}$.

Figure S2 Growth of different colonies from an incubated Bristolian wastewater sample (on the left), result of the incubation for colony no. 7 and no.8 (on the centre), pointing of the single colony to be used a reference (on the right).

Figure S3 Result of the incubation for the colonies found from every day of the sampling campaign in Bristol.

Figure S4 Result of the incubation for the colonies found from every day of the sampling campaign in Oslo, Castellon, Lyngby, Milan, Utrecht and Zurich.

Table S2 Results on colony morphology assessment for the selected cities in the study.

Figure S5 qPCR-Melt curve.

Figure S6 qPCR-Standard curve.

## 7.8 References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# Chapter 8: Conclusions and Future Works

## 8.1 Conclusions

WBE is an innovative approach complementary to more traditional epidemiological techniques, which provides a real-time profiling of community health and lifestyle, through the detection and quantification of biomarkers. Since many drugs are chiral, the investigation on enantiomeric profiling of chiral biomarkers provides a new dimension to WBE. To aid enantiomeric profiling in WBE, the development and validation of two new enantioselective methodologies were performed by chiral HPLC-MS/MS and enabled the chemical analysis of chiral biomarkers.

These novel methodologies were applied to Europe-wide environmental monitoring for:

(1) The estimation of illicit/licit drug use via WBE. The impact of enantiomeric analysis was used for verifying potency, origin and route of administration of drugs of abuse, monitoring any changes in their pattern of use and distinguishing legal from illicit use.

(2) The estimation of quinolones' use via WBE. Since their wide consumption and misuse rapidly brought to an increasing antibiotic resistance, biomarkers of exposure to these antibiotics represented a useful target for understanding any possible misuse in the monitored areas.

The first method investigated 56 human biomarkers for the detection of illicit drugs and potentially abused licit drugs. These were: opioids, amphetamines, cocaine, heroin, stimulants, anaesthetics, sedatives, anxiolytics, designer drugs, PDE5 inhibitors, amphetamine and methamphetamine drug precursors. Satisfactory enantiomeric separation was obtained in a single chromatographic run using a CBH chiral column for 18 pairs of enantiomers including amphetamine, methamphetamine, MDMA and its metabolites HMA and HMMA, PMA, MDA and mephedrone. The method showed very good performance: >90% SPE recoveries, very good sensitivity (MDLs and MQLs at ppt levels), high linearity range and method precision <10%. The first application of this methodology was in a week monitoring campaign in the UK. To the author's knowledge, the enantiomeric profiling of mephedrone and MDMA metabolites was documented for the first time. A predominance of *R*-(+)-mephedrone was found in environmental samples, thus suggesting a possible stereoselective human metabolism and excretion profile. As a result of the investigation of the temporal trend, mephedrone was defined as a recreational drug of abuse due to high loads in the weekend (as in the case of MDMA). A comparable trend was also observed for MDMA and its metabolite HMMA.

After the detection of mephedrone loads in two consecutive sampling campaigns in the UK in 2014 and 2015, a case study on mephedrone posed the basis for a novel approach towards biomarkers' selection in estimation of human exposure to chiral drugs with limited metabolism data. This newly-developed framework consisted of four steps: (i) the identification of possible metabolic biomarkers present in wastewater through in-direct *in-vivo* study using LC-HRMS; (ii) the verification of chiral signature of the target compound using chiral LC-MS/MS; (iii) the

confirmation of human metabolic residues in *in-vivo* and *in-vitro* studies and (iv) the verification of stability of possible biomarkers in wastewater. At the end, mephedrone was chosen as a suitable biomarker due to its high stability in wastewater. Its enantiomeric profiling was studied, for the first time, in several biological and environmental matrices and it showed that chiral analysis was fundamental for distinguishing between human consumption (favouring *R*-(+)-enantiomer) and direct disposal of unused mephedrone (as it was found to be distributed as racemate in the UK). Indeed, *R*-(+)-enantiomer was observed in pHLM experiments and in pooled urine analysis, whilst *S*-(-)-form was favoured *in-vivo* rat metabolism studies. Further biomarker candidates, such as 4'-carboxy-mephedrone, 4'-carboxy-normephedrone, 1-dihydro-mephedrone, 1-dihydro-normephedrone and hydroxyl-tolyl-normephedrone, were also suggested for WBE approach for future studies.

The second method was developed to undertake wastewater profiling for antibiotics in order to understand the spatial and temporal antibiotics' use and the prevalence of resistance genes. In particular, this methodology explored 16 biomarkers of quinolones' use, as they represent one class of antibiotics with rising concern in antibiotic resistance. With >70% SPE recoveries, very good sensitivity, high linearity range and method precision < 20%, it allowed for the analysis of 16 human and veterinary quinolones drugs as potential biomarkers in wastewater. To the author's knowledge, this was the first time that (i) chiral separation of the following biomarkers was simultaneously performed in reverse phase LC-MS/MS using an OZ-RH column: ofloxacin with its main metabolites ofloxacin-*N*-oxide and desmethyl-ofloxacin, moxifloxacin, the prodrug prulifloxacin with its active compound ulifloxacin, *cis*-ketoconazole, flumequine, nadifloxacin and *R*-(+)-besifloxacin and (ii) their enantiomeric profiling was investigated at enantiomeric level in wastewater. As some achiral quinolones were included in this research, the proposed method was also suitable for monitoring purposes. Thus, the most comprehensive panel of quinolones biomarkers was considered for WBE applications.

Both methodologies were applied to wastewaters from eight locations across Europe [i.e. Oslo (Norway), Bristol (United Kingdom), Lyngby (Denmark),

Utrecht (The Netherlands), Brussels (Belgium), Zurich (Switzerland), Milan (Italy) and Castellon (Spain)], thus allowing the first pan-European studies on enantiomeric profiling of chiral biomarkers. This led to an understanding of: (i) new patterns of emerging drugs of abuse, (ii) changes in patterns of classical illicit drugs and quinolones with the verification of the origin of drug residue, potency of abused drug and its synthetic route and (iii) quinolones metabolic profiles. The results indicated that, a new drug of abuse, mephedrone, was prevalent only in the UK (see before). A spatial difference in loads was observed between Northern and Southern European cities for amphetamine, with a slight enrichment of *R*-(-)-amphetamine in wastewater (with the exception of some days in Zurich). Still not well assessed remained the interpretation of the enantiomeric composition of amphetamine in European wastewater samples. High methamphetamine loads were found in Norwegian samples; this is relevant as in Norway high seizures were also seen according to the EMCDDA. Wastewater was enriched of *S*-(+)-methamphetamine probably due its stereoselective synthesis in the illicit manufacturing market, with the exception of Oslo, where a different illegal synthetic route was assumed. The prevalence of *R*-(-)-MDMA in wastewaters was linked to the MDMA consumption, even in those cities where illicit manufacturing sites were found in the past. *S*-(+)-MDA originated mostly from MDMA metabolism. HMMA appeared to be a suitable MDMA's DTR. It was found enriched of the *S*-(+)-enantiomer in wastewaters, suggesting MDMA abuse. The investigation of precursors showed that their presence was reasonably ascribed to their medical use.

Through the investigation of the occurrence of ciprofloxacin and ofloxacin metabolites, it was possible to get a urinary excretion profiling reflected in the wastewater. Higher loads were found in Southern European cities than in northern ones. Enantiomeric profiling revealed the drug residue origin. Indeed, enantiomerically pure *S*-(-)-ofloxacin was observed in Southern locations, whilst racemic ofloxacin was more predominant in Northern European ones. Moxifloxacin's human intake was demonstrated by the presence of *S,S*-moxifloxacin and *S,S*-moxifloxacin-*N*-sulphate. Enantiomeric profiling of prulifloxacin showed that ulifloxacin was present because of prulifloxacin metabolism. No disposal of prulifloxacin was observed. Potential enantioselective metabolism was hypothesised for (±)-*cis*-Ketoconazole due to an enrichment of the first-eluting enantiomer. The enrichment of the first-eluting flumequine enantiomer

was assumed to result from animals' metabolism rather than its direct disposal. Furthermore, the occurrence of quinolones in wastewater was in agreement with estimated quinolones use from ECDC in 2014.

In conclusion, once biomarkers of exposure were detected, biomarkers of effect were identified for specific health-related biomarkers through the usage of bioanalytical techniques, including DNA extraction methods followed by qPCR- and digital PCR-based techniques. Thus, this work led to an analysis of biomarkers of microbial resistance and biomarkers of bacterial infections loads.

## 8.2 Future Work

The results of this research on the determination of wastewater profiling for antibiotics led to a better understanding of both spatial and temporal antibiotics' use and prevalence of resistance genes across Europe. This work served as a proof-of-concept for future research studies aimed at verifying any correlation between the occurrence of biomarkers of exposure and the spreading of biomarkers of antibiotic resistance at local and national level. This will be in support of monitoring activities of antimicrobial resistance. Moreover, as many antibiotics are chiral, further works will be focused on the development of antimicrobial resistance at stereoisomeric level for other classes of antibiotics. This epidemiological approach has the potential to become an early warning system for outbreaks of disease and a unique tool for the identification of hot-spots in the context of antimicrobial resistance.

However, there are still many open questions with antibiotic resistance genes (ARGs) that need to be addressed. ARGs can persist in the environment for an extended period of time and spread among bacteria [1]. Therefore, they can be considered to be emerging environmental "contaminants" as defined by Pruden et al. [2], and they have the potential to be further distributed to various environmental compartment.

Since the antibiotic resistance could spread at every level during the wastewater treatment, the proposed methodology, which combines the detection of antibiotics through analytical techniques and the occurrence of genes through bioanalytical methods, can be applied to other environmental matrices, such as

effluent wastewater and receiving waters. This will allow verifying the mechanisms of transformation of chiral antimicrobial agents and their metabolites during wastewater treatment such as the evaluation of the ofloxacin's metabolic pattern.

## 8.3 Publications and PhD activities

A proven record of the research activities was reflected by a number of peer-reviewed publications at international journals and presentations at international conferences.

In particular, scientific publications were the following:

1. Castrignanò E., Lubben A., Kasprzyk-Hordern B. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. J Chromatogr A, 1438:84-99 (DOI: 10.1016/j.chroma.2016.02.0150), 2016.

2. Yang Z., Castrignanò E., Estrela P., Frost C.G. & Kasprzyk-Hordern B. Community Sewage Sensors towards Evaluation of Drug Use Trends: Detection of Cocaine in Wastewater with DNA-Directed Immobilization Aptamer Sensors. Sci Rep (DOI: 10.1038/srep21024), 2016.

3. Petrie B., Camacho-Munoz D., Castrignanò E., Evans S., Kasprzyk-Hordern B. Chiral Liquid Chromatography Coupled with Tandem Mass Spectrometry for Environmental Analysis of Pharmacologically Active Compounds LC GC EUROPE 28 (3), 151-160, 2015.

4. Camacho-Munoz D., Petrie B., Castrignanò E. and Kasprzyk-Hordern B. Enantiomeric Profiling of Chiral Pharmacologically Active Compounds in the Environment with the usage of chiral Liquid Chromatography Coupled with Tandem Mass Spectrometry. Curr Anal Chem, 12, 2015.

Papers under preparation include (tentative titles are provided):

1. "Enantiomeric profiling of illicit drugs in a pan-European study" (with co-authorship of SEWPROF members),

2. "A new approach towards biomarker selection in estimation of human exposure to chiral drugs with limited metabolism data: a case study of mephedrone" (with co-authorship of Mardal M., Rydevik A., Miserez B., Ramsey J., Shine T., Pantos G. D., Meyer M. R. and Kasprzyk-Hordern B.)

3. “Multi-residue stereoisomeric analysis of human and veterinary chiral drugs in wastewater using chiral liquid chromatography coupled with tandem mass spectrometry”,

4. “Enantiomeric profiling of quinolones and monitoring of resistance genes in European wastewaters”.

The author contributed to collaborative work across the SEWPROF network:

1. Gonzalez-Marino I., Gracia-Lor E., Rousis N., Castrignanò E., Thomas K. V., Quintana J. B., Kasprzyk-Hordern B., Zuccato E. and Castiglioni S. Wastewater-based epidemiology to monitor synthetic cathinones use in different European countries. In: Environ Sci Technol (DOI: 10.1021/acs.est.6b02644).

2. Bade R., Bijlsma L., Sancho J. V., Baz-Lomba J., Castiglioni S., Castrignanò E., Causanilles A., Gracia-Lor E., Kasprzyk-Hordern B., Kinyua J., McCall A., van Nuijs A. L. N., Ort C., Plósz B. G, Ramin P., Rousis N., Ryu Y., Thomas K. V.; de Voogt P., Zuccato E. and Hernandez F. Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities. In: Chemosphere (submitted).

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1. Castrignanò E. and Kasprzyk-Hordern, B. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. In: Testing the Waters

2015: 2nd International Conference on Wastewater-based Drug Epidemiology, 2015-10-11 - 2015-10-15, Ascona, Switzerland.

2. Yang, Z., Kasprzyk-Hordern, B., Angls d'Auriac, M., Goggins, S., Castrignanò E., Rice, J., Thomas, K. V., Frost, C. and Estrela, P., 2015. Community Sensors for Monitoring of Public Health by Means of Wastewater-Based Epidemiology. In: Testing the Waters 2015: 2nd International Conference on Wastewater-based Drug Epidemiology, 2015-10-11 - 2015-10-15, Ascona, Switzerland.

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4. Yang, Z., Angls d'Auriac, M., Goggins, S., Castrignanò E., Rice, J., Estrela, P., Frost, C., Thomas, K. V. and Kasprzyk-Hordern, B. Community Sensors for Monitoring Public Health using Wastewater-Based Epidemiology. In: 15th EuCheMS International Conference on Chemistry and the Environment, 2015-09-20- 2015-09-25, Leipzig, Germany.

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6. Castrignanò E. Programme Working Group Meetings COST Action ES1307. Wastewater-based epidemiology for community-wide antibiotics use assessment. "Sewage biomarker analysis for community health assessment", 27th-28th October 2014, San Anton, Malta.

Finally, a number of presentations were held at the following SEWPROF training courses:

- 2nd SEWPROF training Course Oslo (Norway) in September 2013;
- 3rd SEWPROF training Course in Utrecht (The Netherlands) in April 2014;

- 4th SEWPROF training Course/Mid-Term Review Meeting in Milan (Italy) in September 2014;
- 5th SEWPROF training Course in Castellon (Spain) in March 2015.

## 8.4 References

1. Poté, J., et al., *Fate and transport of antibiotic resistance genes in saturated soil columns.* Eur J Soil Biol, 2003. **39**(2): p. 65-71.
2. Pruden, A., et al., *Antibiotic resistance genes as emerging contaminants: studies in northern Colorado.* Environ Sci Technol, 2006. **40**(23): p. 7445-7450.

# Appendix 1

The following supplementary data are contained in Appendix 1:

Table S1 Selected analytes and their properties.

Table S2 Studied mobile phase compositions with CHIRALPAK® CBH HPLC.

Table S3 Studied mobile phase compositions with CHIROBIOTIC V.

Table S4 Studied mobile phase compositions with CHIROBIOTIC T.

Table S5 Validation parameters -instrumental precision.

Table S6 Validation parameters- ion suppression.

Figure S1 CBH column - enantiomeric resolution of studied analytes in a mobile phase containing acetonitrile as organic modifier (mobile phase composition: 1mM ammonium acetate/acetonitrile 9:1).

Figure S2 CBH column - enantiomeric resolution of studied analytes in a mobile phase containing isopropanol as organic modifier (mobile phase composition: (a)

1mM ammonium acetate/isopropanol 9:1 and (b) 1mM ammonium acetate/isopropanol 9.5:0.5).

Figure S3 CBH column - enantiomeric resolution of studied analytes in mobile phases containing: (a) 1 mM ammonium acetate/methanol 9.5:0.5, (b) 1 mM ammonium acetate/methanol 9:1, (c) 2.5 mM ammonium acetate/methanol 9:1, (d) 5 mM ammonium acetate /methanol 9:1 and (e) 10 mM ammonium acetate /methanol 9:1.

Figure S4 CBH column - Impact of different percentanges of modifiers on retention time of analytes.

Figure S5 Chirobiotic T column - overview of the separation for the targeted analytes.

Figure S6 Chirobiotic T column - separation of oxazepam and lorazepam.

**Table S1** Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, [a] extracted from [38] , [b] predicted using ACD/labs software (http://www.chemspider.com).

| Compound | CAS | Formula | MW | $pK_a$ | | LogP | | LogD[b] | | Supplier |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Exp.[a] | Pred.[a] | Exp.[a] | Pred.[b] | pH 5.5 | pH 7.4 | |
| Cocaine | 50-36-2 | $C_{17}H_{21}NO_4$ | 303.4 | 8.6 (15°) | 8.8 | 2.3[c] | 3.1±0.4 | 0.1 | 1.5 | LGC (Cerilliant product) |
| Benzoylecgonine | 519-09-5 | $C_{16}H_{19}NO_4$ | 289.3 | - | 3.1, 9.5 | - | 2.7±0.4 | 0.2 | 0.2 | Sigma-Aldrich |
| Cuscohygrine | 454-14-8 | $C_{13}H_{24}N_2O$ | 224.3 | - | - | - | 0.7±0.3 | -3.4 | -3.2 | TRC |
| Cocaethylene | 529-38-4 | $C_{18}H_{23}NO_4$ | 317.4 | - | - | - | 2.8 | -0.2 | 1.1 | Sigma Aldrich (Cerilliant product) |
| Anhydroecgonine methyl ester | 43021-26-7 | $C_{10}H_{15}NO_2$ | 181.2 | - | - | - | 1.7±0.3 | -0.7 | 1.0 | Sigma Aldrich (Cerilliant product) |
| (±)-Amphetamine | 300-62-9 | $C_9H_{13}N$ | 135.2 | 10.1 (20°) | 10.0 | 1.8 | 1.8±0.2 | -1.3 | -0.6 | LGC(Cerilliant product) |
| (±)-Methamphetamine | 4846-07-5 | $C_{10}H_{15}N$ | 149.2 | 9.9 (25°) | 10.2 | 2.1 | 1.9±0.2 | -1.1 | -0.8 | LGC (Cerilliant product) |
| *S*-(+)- Methamphetamine | 537-46-2 | $C_{10}H_{15}N$ | 149.2 | 9.9 (25°) | 10.2 | 2.1 | 1.9±0.2 | -1.1 | -0.8 | Cerilliant |
| BZP (benzylpiperazine) | 2759-28-6 | $C_{11}H_{16}N_2$ | 176.3 | - | - | - | 1.4±0.4 | -1.6 | -0.4 | LGC |
| TFMPP (1-(3-trifuoromethylphenyl)piperazine) | - | $C_{11}H_{13}F_3N_2$ | 230.2 | - | - | - | 2.4±0.5 | -0.4 | 1.2 | LGC |
| (±)-Mephedrone | 1189726-22-4 | $C_{11}H_{15}NO$ | 177.7 | - | - | - | 1.9±0.3 | -0.0 | 1.5 | Sigma-Aldrich (Cerilliant product) |
| PMA (*p*-Methoxyamphetamine) | 3706-26-1 | $C_{10}H_{15}NO$ | 165.0 | - | - | - | 1.7±0.2 | -1.4 | -0.8 | LGC |
| (±)-MDA (3,4-methylenedioxyamphetamine) | 4764-17-4 | $C_{10}H_{13}NO_2$ | 179.2 | 9.7 (25°) | 10.0 | 1.6 | 1.7±0.3 | -1.4 | -0.8 | LGC (Cerilliant product) |
| (±)-MDMA (3,4-methylenedioxymethamphetamine) | 42542-10-9 | $C_{11}H_{15}NO_2$ | 193.2 | - | 10.1 | -1.6,1.9 | 1.8±0.3 | -1.3 | -0.9 | LGC |
| (±)-MDEA (3,4-methylenedioxyethylamphetamine) | 82801-81-8 | $C_{12}H_{17}NO_2$ | 207.3 | - | - | - | 2.7±0.3 | -0.4 | 0.3 | LGC (Cerilliant product) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *D,L*-HMA (d,l-4-Hydroxy-3-methoxyamphetamine) | 13062-61-8 | $C_{10}H_{15}NO_2$ | 181.2 | - | - | - | - | - | - | Kinesis |
| *D,L*-HMMA (d,l-4-Hydroxy-3-methoxymethamphetamine) | 438625-58-2 | $C_{11}H_{17}NO_2$ | 195.2 | - | - | - | 1.4 | -1.7 | -1.2 | Kinesis |
| *D,L*-3,4-HHMA (2-(3,4-Dihydroxyphenyl)-*N*-methylpropylamine) | 15398-87-5 | $C_{10}H_{15}NO_2$ | 181.2 | - | - | - | - | - | - | Kinesis |
| Caffeine | 58-08-2 | $C_8H_{10}N_4O_2$ | 194.2 | 10.4 (40°) | -0.9 | -0.1 | -0.1±0.4 | -0.1 | -0.1 | Sigma-Aldrich |
| 1,7-dimethylxanthine (Paraxanthine) | 611-59-6 | $C_7H_8N_4O_2$ | 180.2 | - | - | - | -1.6±0.9 | -1.6 | -1.6 | Sigma-Aldrich |
| (-)-Nicotine | 54-11-5 | $C_{10}H_{14}N_2$ | 162.2 | 3.1 | 8.9 | 1.2 | 0.7±0.3 | -2.1 | -0.5 | Sigma-Aldrich |
| (-)-Cotinine | 486-56-6 | $C_{10}H_{12}N_2O$ | 176.2 | - | - | - | -0.2±0.4 | -0.3 | -0.2 | Sigma Aldrich (Cerilliant product) |
| Heroin | 561-27-3 | $C_{21}H_{23}NO_5$ | 369.4 | 7.9 (25°) | 9.1 | 1.6 | 1.5±0.7 | -0.8 | 0.9 | Sigma Aldrich (Cerilliant product) |
| 6-acetylmorphine | 2784-73-8 | $C_{19}H_{21}NO_4$ | 327.4 | - | 10.2, 9.1 | 1.9,1.3 | 0.4±0.7 | -1.7 | -0.1 | Sigma Aldrich (Cerilliant product) |
| Codeine | 76-57-3 | $C_{18}H_{21}NO_3$ | 299.4 | 8.2 (25°) | 13.8, 9.2 | 1.2, 1.3 | 1.2±0.7 | -1.4 | 0.3 | Sigma-Aldrich |
| Norcodeine | 467-15-2 | $C_{17}H_{19}NO_3$ | 285.3 | - | 13.8, 10.1 | 1.0, 1.0 | 0.9±0.7 | -2.1 | -1.1 | Sigma Aldrich (Cerilliant product) |
| Oxycodone | 76-42-6 | $C_{18}H_{21}NO_4$ | 315.4 | - | 13.6, 8.2 | 1.0, 1.0 | 1.7±0.6 | -0.5 | 1.2 | Sigma Aldrich (Cerilliant product) |
| Noroxycodone | 52446-25--0 | $C_{17}H_{19}NO_4$ | 301.2 | - | 13.6, 9.5 | - | 0.2 | -2.7 | -1.1 | Sigma Aldrich (Cerilliant product) |
| (-)-Oxymorphone | 76-41-5 | $C_{17}H_{19}NO_4$ | 301.3 | - | 10.1, 8.2 | - | 0.9±0.5 | -1.1 | 0.5 | Sigma Aldrich (Cerilliant product) |
| *D*-(-)-Morphine | 57-27-2 | $C_{17}H_{19}NO_3$ | 285.3 | 8.2 (25°) | 10.3, 9.1 | 0.9 | 0.4±0.7 | -2.1 | -0.4 | Sigma Aldrich (Cerilliant product) |
| Normorphine | 466-97-7 | $C_{16}H_{17}NO_3$ | 271.3 | - | 10.5, 9.8 | - | 0.1±0.7 | -2.9 | -1.8 | Sigma Aldrich (Cerilliant product) |
| Dihydromorphine | 509-60-4 | $C_{17}H_{21}NO_3$ | 287.4 | - | 10.3, 9.2 | - | 0.6±0.4 | -2.0 | -0.4 | Sigma Aldrich (Cerilliant product) |
| Hydrocodone | 125-29-1 | $C_{18}H_{21}NO_3$ | 299.4 | - | 18.0, 8.6 | 1.2 | 1.8±0.5 | -0.9 | 0.7 | Sigma Aldrich (Cerilliant product) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Morphine-3β-*D*-glucuronide | 20290-09-9 | $C_{23}H_{27}NO_9$ | 461.5 | - | 12.2, 10.8 | - | -2.0±0.8 | -4.5 | -4.6 | Sigma Aldrich (Cerilliant product) |
| (±)-Methadone | 76-99-3 | $C_{21}H_{27}NO$ | 309.4 | 8.9 (25°) | 18.8, 9.1 | 3.9 | 4.2±0.3 | 1.2 | 2.6 | Sigma Aldrich (Cerilliant product) |
| (±)-EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) | 66729-78-0 | $C_{20}H_{23}N$ | 277.4 | - | 9.6 | - | 5.4 | 3.6 | 4.9 | LGC (Cerilliant product) |
| (±)-*cis*-Tramadol | 36282-47-0 | $C_{16}H_{25}NO_2$ | 263.4 | 9.4 | 13.8, 9.2 | 2.4 | 2.5±0.3 | -0.5 | 0.5 | Sigma-Aldrich |
| *N*-Desmethyltramadol | 1018989-94-0 | $C_{15}H_{23}NO_2$ | 249.4 | - | 13.8, 9.9 | - | 1.7 | -1.4 | -1.1 | LGC |
| (+)-*O*-Desmethyltramadol | 185453-02-5 | $C_{15}H_{23}NO_2$ | 249.4 | - | 9.6, 9.0 | - | 1.7 | -1.3 | -0.2 | LGC |
| Temazepam | 846-50-4 | $C_{16}H_{13}ClN_2O_2$ | 300.7 | - | 10.7, -1.4 | 2.2 | 2.1±0.9 | 2.1 | 2.1 | Sigma Aldrich (Cerilliant product) |
| Diazepam | 439-14-5 | $C_{16}H_{13}ClN_2O$ | 284.7 | 3.4 | 2.9 | 2.8 | 2.9±0.9 | 2.9 | 2.9 | Sigma Aldrich (Cerilliant product) |
| Nordiazepam | 1088-11-5 | $C_{15}H_{11}ClN_2O$ | 270.7 | - | 12.3, 2.8 | 2.5[b] | 3.1±0.5 | 3.1 | 3.1 | Sigma Aldrich (Cerilliant product) |
| Nitrazepam | 146-22-5 | $C_{15}H_{11}N_3O_3$ | 281.3 | - | 11.9, 2.6 | 2.2 | 2.2±0.5 | 2.2 | 2.2 | LGC (Cerilliant product) |
| 7-aminonitrazepam | 4928-02-3 | $C_{15}H_{13}N_3O$ | 251.3 | - | - | - | 1.1±0.8 | 1.0 | 1.1 | Sigma Aldrich (Cerilliant product) |
| Oxazepam | 604-75-1 | $C_{15}H_{11}ClN_2O_2$ | 286.7 | - | 10.6, -1.5 | - | 2.3±0.5 | 2.3 | 2.3 | Sigma-Aldrich (Cerilliant product) |
| (±)-Lorazepam | 846-49-1 | $C_{15}H_{10}Cl_2N_2O_2$ | 321.2 | 13 | 10.6, -2.2 | 2.4 | 2.5±0.5 | 2.5 | 2.5 | Sigma-Aldrich (Cerilliant product) |
| Amitriptyline | 549-18-8 | $C_{20}H_{23}N$ | 277.4 | 9.4 | 9.8 | 4.9 | 4.9±0.6 | 1.9 | 3.1 | Sigma-Aldrich |
| Nortriptyline | 894-71-3 | $C_{19}H_{21}N$ | 263.4 | - | 10.5 | - | 5.6±0.3 | 2.6 | 3.2 | Sigma-Aldrich |
| Fluoxetine | 59333-67-4 | $C_{17}H_{18}F_3NO$ | 309.3 | - | 9.8 | 4.0 | 4.1±0.4 | 1.0 | 1.6 | LGC (Cerilliant product) |
| *R*-(-)-fluoxetine | 114247-09-5 | $C_{17}H_{18}F_3NO$ | 309.3 | - | 9.8 | 4.0 | 4.1±0.4 | 1.0 | 1.6 | Sigma-Aldrich |
| Norfluoxetine | 107674-50-0 | $C_{16}H_{16}F_3NO$ | 295.3 | - | 9.8 | - | 4.4±0.4 | 1.4 | 2.7 | LGC (Cerilliant product) |
| (±)-Venlafaxine | 99300-78-4 | $C_{17}H_{27}NO_2$ | 277.4 | - | 14.4, 8.9 | - | 2.9±0.3 | -0.1 | 1.2 | Sigma-Aldrich |
| *O*-Desvenlafaxine | 300827-87-6 | $C_{16}H_{25}NO_2$ | 263.0 | - | 10.1, 8.9 | - | 2.3±0.3 | -0.7 | 0.5 | Sigma-Aldrich |
| Zolpidem | 99294-93-6 | $C_{19}H_{21}N_3O$ | 307.4 | 6.2 | 5.6 | 1.2 | 3.1±0.6 | 1.9 | 3.0 | Sigma Aldrich (Cerilliant product) |
| (±)-Zopiclone | 43200-80-2 | $C_{17}H_{17}ClN_6O_6$ | 388.8 | - | 13.0, 6.9 | 0.8 | -0.3±1.3 | -1.5 | -0.4 | LGC |
| (±)-Ketamine | 1867-66-9 | $C_{13}H_{16}ClNO$ | 237.7 | - | 18.8, 7.4 | 2.9 | 2.2±0.6 | 1.2 | 2.1 | Sigma-Aldrich |
| (±)-Norketamine | 79499-59-5 | $C_{12}H_{14}ClNO$ | 223.7 | - | 18.7, 7.5 | - | 1.9±0.5 | 1.1 | 1.9 | Sigma Aldrich (Cerilliant product) |
| Sildenafil | 139755-83-2 | $C_{22}H_{30}N_6O_4S$ | 474.6 | - | 7.3, 6.0 | 1.9 | 2.3±1.4 | 1.6 | 2.2 | Sigma Aldrich (Cerilliant product) |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Vardenafil | 224789-1515-5 | $C_{23}H_{32}N_6O_4S$ | 488.6 | - | 8.0, 6.2 | 1.4 | 2.6±1.2 | 1.0 | 2.5 | Sigma Aldrich (Cerilliant product) |
| (±)-Pentobarbital | 76-74-4 | $C_{11}H_{18}N_2O_3$ | 226.3 | 8.1 (25°) | 8.5 | 2.1 | 2.0±0.2 | 2.0 | 1.9 | Sigma-Aldrich (Cerilliant product) |
| Secobarbital | 29071-21-4 | $C_{12}H_{18}N_2O_3$ | 238.3 | 7.8 | 8.5 | 1.9 | 2.2±0.2 | 2.2 | 2.0 | Sigma-Aldrich (Cerilliant product) |
| Ephedrine | 50-98-6 | $C_{10}H_{15}NO$ | 165.2 | 10.3 (0°) | 13.9, 9.5 | 1.1 | 1.0±0.3 | -2.0 | -0.9 | Sigma-Aldrich |
| (*1R,2R*)-(-)-Pseudoephedrine | 321-97-1 | $C_{10}H_{15}NO$ | 165.2 | 10.3 (0°) | 13.9, 9.5 | 1.1 | 1.0±0.3 | -2.0 | -0.9 | Sigma-Aldrich |
| (±)-Norephedrine | 154-41-6 | $C_9H_{13}NO$ | 151.2 | 9.4 (20°) | 13.9, 9.4 | 0.7 | 0.8±0.3 | -2.2 | -1.1 | Sigma-Aldrich |

**Table S2** Studied mobile phase compositions with CHIRALPAK® CBH HPLC

| % MP modifiers | Conc. $NH_4OAc$ (mM) | pH |
|---|---|---|
| 10% IPA | 1.0 | 5.0 |
| 10% MeOH | 1.0 | 6.6 |
| 10% ACN | 1.0 | 6.4 |
| 5% IPA | 1.0 | 6.2 |
| 5% MeOH | 1.0 | 6.7 |
| 10% MeOH | 5.0 | 6.8 |
| 10% MeOH | 10.0 | 6.9 |
| 10% MeOH | 1.0 | 6. 7 |
| 10% MeOH | 2.5 | 6.6 |
| 10% MeOH | 1.0 | 6.2 |
| 15% MeOH | 1.0 | 6.4 |

**Table S3** Studied mobile phase compositions with CHIROBIOTIC V

| % $H_2O$ | %FA | Conc. $NH_4OAc$ (mM) | pH |
|---|---|---|---|
| 1 | 0.005 | 4 | 6.8 |
| 5 | 0.005 | 4 | 6.8 |
| 20 | 0.005 | 4 | 6.5 |
| 80 | 0.005 | 4 | 5.2 |
| 25 - 0 | 0.005 | 4 | |
| 0 | 0 | 0 | |
| 1 | 0.005 | 1 | 5.9 |
| 1 | 0.005 | 10 | 7.4 |
| 1 | 0.001 | 1 | 7.3 |

**Table S4** Studied mobile phase compositions with CHIROBIOTIC T (mobile phases with pH<3 were not tested due to the extreme pH not suitable for the studied chiral column)

| % $H_2O$ | %FA | Conc. $NH_4OAc$ (mM) | pH |
|---|---|---|---|
| 1 | 0.005 | 4 | 6.8 |
| 0 | 0 | 0 | |
| 5 | 0.005 | 4 | 6.8 |
| 20 | 0.005 | 4 | 6.5 |
| 80 | 0.005 | 4 | 5.2 |
| 80 | 0 | 20 | 6.7 |
| 0 - 100 | 0.005 | 4 | |
| 1 | 0.005 | 1 | 5.9 |
| 1 | 0.005 | 10 | 7.4 |
| 1 | 0.001 | 1 | 7.3 |
| 1 | 0.001 | 10 | 7.9 |
| 1 | 0.001 | 4 | 7.6 |
| 1 | 0.01 | 1 | 5.2 |
| 1 | 0.01 | 10 | 6.9 |
| 1 | 0.01 | 4 | 6.4 |
| 1 | 1 | 1 | 2. 9 |
| 1 | 1 | 10 | 3.9 |
| 1 | 1 | 4 | 3.5 |
| 5 | 0.005 | 1 | 5.6 |
| 5 | 0.005 | 10 | 7.0 |

**Table S5** Validation parameters -instrumental precision

| | Intra-day RSD% (n=4) | | | | | | | | | Inter-day RSD% (n=3) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 µg/L** D 1* | 5 µg/L D 2 | 5 µg/L D 3 | 50 µg/L D 1 | 50 µg/L D 2 | 50 µg/L D 3 | 500 µg/L D 1 | 500 µg/L D 2 | 500 µg/L D 3 | 5 µg/L | 50 µg/L | 500 µg/L |
| Cocaine | 1.2 | 2.3 | 5.3 | 2.2 | 4.0 | 0.5 | 3.2 | 3.7 | 0.5 | 2.9 | 2.3 | 2.5 |
| Benzoylecgonine | 3.1 | 4.1 | 2.6 | 6.2 | 2.9 | 2.7 | 1.1 | 1.8 | 1.7 | 3.3 | 3.9 | 1.5 |
| Cocaethylene | 7.9 | 3.5 | 4.8 | 0.2 | 0.4 | 0.6 | 0.2 | 3.8 | 2.4 | 5.4 | 0.4 | 2.1 |
| *R*-(-)-Amphetamine | 4.8 | 5.8 | 3.0 | 2.3 | 3.1 | 0.1 | 3.9 | 4.7 | 3.1 | 4.5 | 1.9 | 3.9 |
| *S*-(+)-Amphetamine | 3.7 | 5.3 | 6.5 | 4.6 | 3.3 | 4.3 | 3.2 | 4.1 | 3.4 | 5.2 | 4.1 | 3.6 |
| *R*-(-)-Methamphetamine | 6.0 | 5.8 | 6.3 | 3.9 | 5.5 | 2.3 | 3.0 | 5.1 | 2.8 | 6.0 | 3.9 | 3.7 |
| *S*-(+)-Methamphetamine | 2.4 | 2.3 | 7.7 | 2.7 | 0.7 | 2.1 | 1.1 | 4.8 | 3.4 | 4.1 | 1.8 | 3.1 |
| E1-Mephedrone | 9.3 | 6.7 | 5.5 | 1.9 | 5.7 | 5.4 | 2.9 | 5.5 | 4.4 | 7.1 | 4.3 | 4.3 |
| E2-Mephedrone | 3.5 | 6.7 | 1.1 | 3.6 | 2.5 | 2.7 | 9.3 | 4.3 | 2.2 | 3.8 | 3.0 | 5.2 |
| *R*-(-)-MDA | 6.9 | 1.3 | 2.7 | 0.4 | 5.6 | 0.1 | 1.5 | 0.3 | 1.6 | 3.6 | 2.1 | 1.1 |
| *S*-(+)-MDA | 5.7 | 3.2 | 6.4 | 8.0 | 8.9 | 3.1 | 0.3 | 1.1 | 6.1 | 5.1 | 6.7 | 2.5 |
| *R*-(-)-MDMA | 2.5 | 5.5 | 2.0 | 1.8 | 6.4 | 3.9 | 4.8 | 3.7 | 6.1 | 3.3 | 4.0 | 4.9 |
| *S*-(+)-MDMA | 3.5 | 1.1 | 4.3 | 0.5 | 1.8 | 1.3 | 2.5 | 1.5 | 2.7 | 3.0 | 1.2 | 2.3 |
| E1-MDEA | 8.6 | 5.3 | 5.9 | 2.2 | 3.8 | 1.1 | 6.1 | 4.3 | 0.1 | 6.6 | 2.4 | 3.5 |
| E2-MDEA | 3.6 | 2.3 | 10.3 | 5.3 | 1.1 | 0.4 | 5.6 | 1.9 | 0.7 | 5.4 | 2.3 | 2.7 |
| Heroin | 6.7 | 17.8 | 0.0 | 2.4 | 11.3 | 29.2 | 11.5 | 13.9 | 13.4 | 8.2 | 14.3 | 13.0 |
| *O*-6-monoacetylmorphine | 9.0 | 9.2 | 12.4 | 0.0 | 1.8 | 11.0 | 1.2 | 10.7 | 1.4 | 10.2 | 4.3 | 4.4 |
| Morphine | 12.1 | 4.4 | 10.6 | 13.5 | 5.5 | 7.9 | 11.0 | 1.2 | 4.4 | 9.1 | 8.9 | 5.5 |
| Morphine-3β-*D*-glucuronide | 14.8 | 19.9 | 2.5 | 4.6 | 16.8 | 20.0 | 8.3 | 6.6 | 13.3 | 12.4 | 13.8 | 9.4 |
| Ketamine | 4.0 | 5.4 | 7.1 | 2.0 | 1.4 | 1.9 | 1.3 | 1.8 | 1.6 | 5.5 | 1.8 | 1.5 |
| Benzylpiperazine | 6.8 | 2.4 | 2.2 | 6.6 | 2.5 | 10.8 | 1.1 | 1.8 | 3.1 | 3.8 | 6.6 | 2.0 |
| Temazepam | 5.9 | 7.2 | 7.9 | 0.7 | 2.2 | 6.4 | 4.4 | 4.1 | 0.3 | 7.0 | 3.1 | 2.9 |
| Diazepam | 4.9 | 4.8 | 6.3 | 1.2 | 2.8 | 4.4 | 2.3 | 1.7 | 1.3 | 5.3 | 2.8 | 1.8 |
| Nordiazepam | 7.0 | 8.4 | 7.3 | 5.1 | 7.5 | 3.6 | 2.2 | 1.6 | 7.4 | 7.6 | 5.4 | 3.7 |
| Nitrazepam | 7.1 | 4.2 | 0.8 | 7.3 | 7.8 | 2.9 | 8.5 | 5.6 | 4.7 | 4.0 | 6.0 | 6.3 |
| Oxazepam | 7.5 | 7.7 | 7.3 | 2.7 | 5.5 | 0.0 | 1.7 | 1.6 | 0.6 | 7.5 | 2.7 | 1.3 |
| 7-amino-nitrazepam | 3.4 | 3.6 | 6.5 | 4.7 | 3.3 | 1.8 | 4.7 | 5.0 | 0.8 | 4.5 | 3.2 | 3.5 |
| Lorazepam | 1.9 | 8.4 | 21.2 | 7.5 | 6.9 | 1.6 | 4.8 | 6.6 | 7.2 | 10.5 | 5.3 | 6.2 |
| AEME | 6.8 | 4.2 | 2.2 | 4.4 | 2.9 | 4.0 | 0.7 | 6.5 | 2.0 | 4.4 | 3.8 | 3.1 |
| E1-HMA | 11.3 | 5.6 | 6.3 | 5.3 | 6.7 | 9.1 | 7.4 | 4.9 | 2.1 | 7.7 | 7.1 | 4.8 |
| E2-HMA | 6.1 | 1.7 | 1.1 | 3.1 | 0.4 | 2.5 | 8.9 | 6.3 | 9.0 | 3.0 | 2.0 | 8.1 |
| E1-HMMA | 5.3 | 8.3 | 4.1 | 0.8 | 6.5 | 6.6 | 8.2 | 4.2 | 1.7 | 5.9 | 4.6 | 4.7 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E2-HMMA | 6.6 | 5.7 | 9.4 | 2.4 | 3.3 | 7.4 | 3.8 | 4.0 | 4.6 | 7.2 | 4.4 | 4.1 |
| DHMA | 8.1 | 4.5 | 9.2 | 3.2 | 12.3 | 5.4 | 3.5 | 2.6 | 1.7 | 7.3 | 7.0 | 2.6 |
| Caffeine | 1.9 | 0.9 | 4.4 | 3.0 | 12.9 | 4.4 | 4.5 | 3.6 | 1.3 | 2.4 | 6.7 | 3.1 |
| 1,7-dimethylxanthine | 1.5 | 8.0 | 7.7 | 2.8 | 15.3 | 6.6 | 0.2 | 0.3 | 0.9 | 5.7 | 8.2 | 0.5 |
| Nicotine | 1.6 | 9.8 | 8.6 | 2.7 | 13.4 | 2.7 | 1.7 | 1.6 | 1.8 | 6.7 | 6.3 | 1.7 |
| Cotinine | 1.1 | 2.6 | 6.5 | 7.0 | 11.8 | 2.4 | 0.1 | 1.9 | 4.1 | 3.4 | 7.1 | 2.0 |
| Creatinine | 5.2 | 6.0 | 7.4 | 2.3 | 0.1 | 1.2 | 1.1 | 0.8 | 1.6 | 6.2 | 1.2 | 1.2 |
| Codeine | 11.7 | 7.3 | 2.3 | 7.6 | 1.7 | 6.9 | 6.0 | 2.6 | 4.8 | 7.1 | 5.4 | 4.5 |
| Oxycodone | 8.3 | 7.3 | 4.0 | 2.5 | 10.5 | 6.6 | 5.6 | 8.8 | 5.3 | 6.5 | 6.5 | 6.6 |
| Noroxycodone | 1.91 | 3.3 | 4.0 | 6.0 | 2.3 | 3.8 | 1.4 | 5.8 | 2.0 | 3.1 | 4.0 | 3.1 |
| Hydrocodone | 3.8 | 5.2 | 4.7 | 1.3 | 7.8 | 4.1 | 2.0 | 0.6 | 1.3 | 4.6 | 4.4 | 1.3 |
| Oxymorphone | 7.3 | 6.8 | 5.5 | 0.4 | 4.5 | 2.8 | 2.1 | 5.9 | 2.1 | 6.5 | 2.6 | 3.4 |
| Dihydrocodeine | 6.8 | 8.2 | 7.4 | 6.6 | 8.7 | 3.4 | 0.5 | 2.0 | 0.6 | 7.4 | 6.2 | 1.0 |
| Methadone | 0.6 | 2.1 | 1.6 | 2.5 | 1.0 | 3.1 | 1.3 | 0.9 | 0.2 | 1.4 | 2.2 | 0.8 |
| EDDP | 5.8 | 4.8 | 5.1 | 1.7 | 2.7 | 2.2 | 0.5 | 1.3 | 1.5 | 5.2 | 2.2 | 1.1 |
| E1-Venlafaxine | 7.1 | 3.1 | 7.4 | 3.4 | 2.0 | 0.8 | 0.4 | 1.4 | 0.9 | 5.9 | 2.1 | 0.9 |
| E2-Venlafaxine | 6.1 | 2.4 | 0.0 | 1.9 | 2.9 | 3.6 | 0.5 | 1.4 | 0.9 | 2.8 | 2.8 | 0.9 |
| Vardenafil | 25.0 | 7.3 | 21.8 | 5.1 | 1.1 | 5.1 | 0.1 | 0.8 | 3.1 | 18.0 | 3.8 | 1.3 |
| E1-Norephedrine | 5.9 | 7.1 | 3.1 | 2.7 | 6.9 | 5.7 | 2.2 | 1.3 | 1.1 | 5.4 | 5.1 | 1.5 |
| E2-Norephedrine | 5.4 | 3.1 | 4.4 | 2.4 | 4.7 | 3.6 | 3.4 | 5.2 | 2.1 | 4.3 | 3.6 | 3.6 |
| E1-PMA | 2.7 | 8.4 | 5.6 | 1.2 | 8.6 | 6.1 | 2.6 | 0.3 | 0.4 | 5.6 | 5.3 | 1.1 |
| E2-PMA | 4.6 | 5.4 | 4.5 | 4.6 | 3.9 | 2.6 | 3.1 | 1.6 | 1.1 | 4.9 | 3.7 | 1.9 |
| Normorphine | 4.6 | 13.3 | 0.0 | 20.0 | 0.5 | 8.2 | 3.8 | 0.1 | 15.8 | 6.0 | 9.5 | 6.6 |
| Dihydromorphine | 18.1 | 3.0 | 5.3 | 15.7 | 20.0 | 4.8 | 8.9 | 4.9 | 11.0 | 8.8 | 13.5 | 8.3 |
| D1-Tramadol | 11.2 | 7.1 | 5.6 | 3.4 | 4.1 | 3.6 | 5.1 | 0.9 | 2.5 | 8.0 | 3.7 | 2.8 |
| D2-Tramadol | 2.4 | 6.8 | 10.7 | 13.8 | 12.4 | 7.1 | 2.2 | 10.0 | 1.1 | 6.7 | 11.1 | 4.4 |
| *O*-desmethyltramadol | 12.9 | 10.9 | 7.2 | 6.2 | 3.0 | 3.5 | 8.3 | 5.2 | 3.2 | 10.3 | 4.2 | 5.6 |
| Zolpidem | 15.7 | 16.3 | 1.6 | 1.2 | 0.1 | 1.1 | 3.3 | 3.4 | 0.2 | 11.2 | 0.8 | 2.3 |
| Amitriptyline | 0.0 | 10.1 | 8.3 | 3.1 | 6.0 | 11.3 | 3.4 | 3.4 | 3.4 | 6.1 | 6.8 | 3.4 |
| Norketamine | 2.7 | 6.9 | 6.0 | 3.4 | 1.1 | 3.5 | 2.6 | 2.4 | 1.4 | 5.2 | 2.7 | 2.1 |
| Sildenafil | 5.4 | 15.7 | 10.9 | 4.8 | 15.3 | 5.7 | 7.6 | 0.9 | 1.5 | 10.7 | 8.6 | 3.3 |
| (+)-Ephedrine | 6.9 | 3.5 | 6.5 | 4.3 | 5.7 | 3.2 | 6.3 | 1.0 | 3.4 | 5.6 | 4.4 | 3.6 |
| (-)-Ephedrine and (-)-Ψephedrine | 2.6 | 2.7 | 4.1 | 3.6 | 3.1 | 2.3 | 6.4 | 0.7 | 4.4 | 3.1 | 3.0 | 3.8 |
| (+)-Ψephedrine | 10.6 | 6.2 | 5.6 | 5.9 | 0.5 | 2.4 | 3.4 | 9.1 | 3.2 | 7.4 | 2.9 | 5.3 |
| Desmethylvenlafaxine-E1 | 18.2 | 7.4 | 5.2 | 5.3 | 7.2 | 0.7 | 1.9 | 8.1 | 2.0 | 10.3 | 4.4 | 4.0 |
| Desmethylvenlafaxine-E2 | 5.4 | 4.5 | 4.0 | 5.7 | 12.5 | 6.4 | 2.8 | 2.0 | 3.9 | 4.7 | 8.2 | 2.9 |
| E1-Zopiclone | 18.2 | 19.7 | 15.8 | 14.2 | 16.2 | 12.2 | 11.4 | 10.7 | 8.7 | 17.9 | 14.2 | 10.3 |
| E2-Zopiclone | 17.9 | 19.3 | 16.7 | 12.6 | 18.6 | 18.0 | 14.6 | 13.8 | 8.5 | 17.9 | 16.4 | 12.3 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S*-(+)-Fluoxetine | 18.3 | 6.9 | 4.9 | 6.0 | 19.0 | 9.3 | 13.6 | 14.4 | 2.4 | 10.0 | 11.4 | 10.2 |
| *R*-(-)-Fluoxetine | 16.5 | 15.9 | 10.2 | 13.0 | 1.3 | 16.9 | 1.7 | 9.3 | 2.4 | 14.2 | 10.4 | 4.5 |
| E1-Norfluoxetine | 11.5 | 4.4 | 18.4 | 5.9 | 8.2 | 0.8 | 5.1 | 3.1 | 3.4 | 11.4 | 5.0 | 3.9 |
| E2-Norfluoxetine | 10.2 | 14.3 | 16.6 | 15.5 | 9.8 | 13.8 | 17.6 | 8.0 | 0.5 | 13.7 | 13.1 | 8.7 |

*-D indicates day

**- the following concentrations were used: 10, 100 and 1000 ng/L in the case of compounds that were not enantioseparated

**Table S6** Validation parameters –ion suppression

| | Signal suppression (%) (n=4) |
|---|---|
| Cocaine | -69.0 ± 25.8 |
| Benzoylecgonine | -6.1 ± 1.5 |
| Cocaethylene | -27.5 ± 5.2 |
| *R*-(-)-Amphetamine | 37.9 ± 9.7 |
| *S*-(+)-Amphetamine | 53.4 ± 9.4 |
| *R*-(-)-Methamphetamine | -28.5 ± 12.5 |
| *S*-(+)-Methamphetamine | -6.4 ± 9.2 |
| E1-Mephedrone | -22.3 ± 11.8 |
| E2-Mephedrone | -40.2 ± 14.0 |
| *R*-(-)-MDA | -15.3 ± 1.4 |
| *S*-(+)-MDA | -12.5 ± 1.8 |
| *R*-(-)-MDMA | -43.9 ± 7.4 |
| *S*-(+)-MDMA | -57.5 ± 8.2 |
| E1-MDEA | -33.9 ± 2.0 |
| E2-MDEA | -58.1 ± 6.6 |
| Heroin | -5.1 ± 10.3 |
| *O*-6-monoacetylmorphine | -85.2 ± 18.8 |
| Morphine | -58.1 ± 18.5 |
| Morphine-3β-*D*-glucuronide | 99.7 ± 0.6 |
| Ketamine | 12.5 ± 11.2 |
| Benzylpiperazine | -50.1 ± 5.3 |
| Temazepam | 21.5 ± 4.3 |
| Diazepam | -37.5 ± 7.7 |
| Nordiazepam | -34.9 ± 1.5 |
| Nitrazepam | 45.7 ± 2.6 |
| Oxazepam | 47.7 ± 4.2 |
| 7-amino-nitrazepam | 70.9 ± 6.7 |
| Lorazepam | 49.3 ± 10.5 |
| Anhydroecgonine methyl ester | -90.4 ± 2.9 |
| E1-HMA | -50.4 ± 6.2 |
| E2-HMA | -68.7 ± 13.9 |
| E1-HMMA | -81.5 ± 33.7 |
| E2-HMMA | -76.7 ± 15.0 |
| DHMA | 95.4 ± 10.1 |
| Caffeine | 57.3 ± 12.3 |
| 1,7-dimethylxanthine | 59.3 ± 9.4 |
| Nicotine | -9.4 ± 7.1 |
| Cotinine | 49.0 ± 10.9 |
| Creatinine | 70.1 ± 2.3 |
| Codeine | -5.2 ± 7.2 |
| Oxycodone | -58.5 ± 11.5 |
| Noroxycodone | -58.6 ± 7.8 |
| Hydrocodone | -50.8 ± 7.6 |
| Oxymorphone | -74.7 ± 10.5 |
| Dihydrocodeine | -6.6 ± 11.6 |
| Methadone | 37.6 ± 19.9 |
| EDDP | 23.9 ± 1.8 |
| E1-Venlafaxine | -19.3 ± 4.8 |
| E2-Venlafaxine | -12.1 ± 9.5 |
| Vardenafil | 15.7 ± 10.8 |
| E1-Norephedrine | 63.4 ± 2.8 |
| E2-Norephedrine | 21.5 ± 4.6 |
| E1-PMA | -21.7 ± 7.9 |
| E2-PMA | -38.8 ± 4.1 |
| Normorphine | 63.2 ± 11.0 |
| Dihydromorphine | 60.0 ± 10.2 |
| D1-Tramadol | 22.1 ± 1.5 |
| D2-Tramadol | 8.8 ± 6.6 |
| *O*-desmethyltramadol | 46.7 ± 3.0 |
| Zolpidem | -72.1 ± 3.2 |
| Amitriptyline | -23.5 ± 0.6 |
| Norketamine | -52.1 ± 2.4 |
| Sildenafil | -49.9 ± 11.8 |

| | |
|---|---|
| (+)-Ephedrine | -78.2 ± 4.6 |
| (-)-Ephedrine and (-)-Ψephedrine | -72.3 ± 7.5 |
| (+)-Ψephedrine | -76.7 ± 16.3 |
| Desmethylvenlafaxine-E1 | -6.3 ± 2.0 |
| Desmethylvenlafaxine-E2 | -31.1 ± 16.4 |
| E1-Zopiclone | -33.2 ± 5.6 |
| E2-Zopiclone | -41.0 ± 4.5 |
| *S*-(+)-Fluoxetine | 1.5 ± 0.1 |
| *R*-(-)-Fluoxetine | 3.2 ± 2.5 |
| E1-Norfluoxetine | -4.3 ± 0.7 |
| E2-Norfluoxetine | -11.0 ± 0.8 |

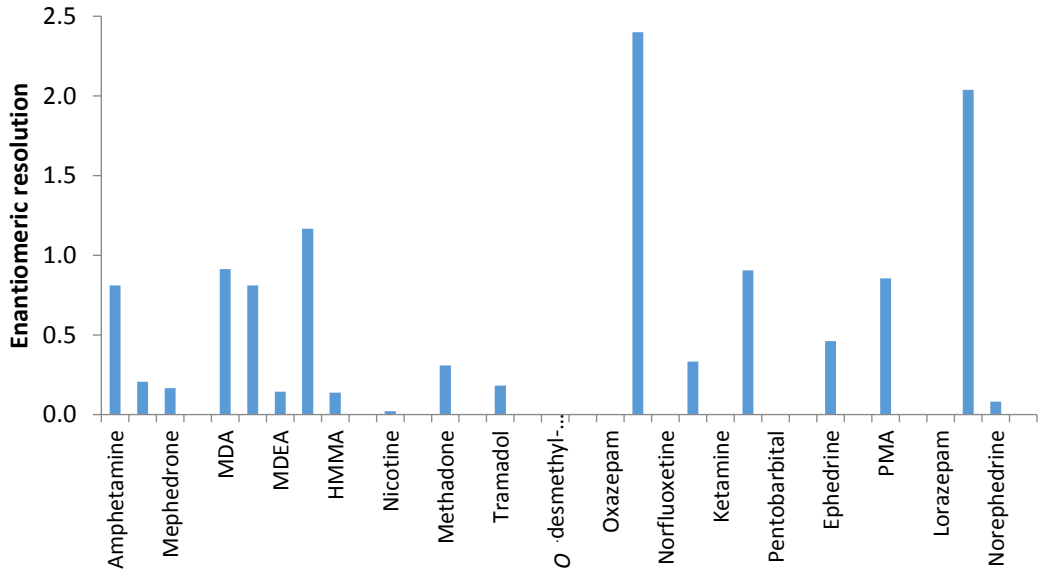



**Figure S1** CBH column - **e**nantiomeric resolution of studied analytes in a mobile phase containing acetonitrile as organic modifier (mobile phase composition: 1mM ammonium acetate/acetonitrile 9:1).

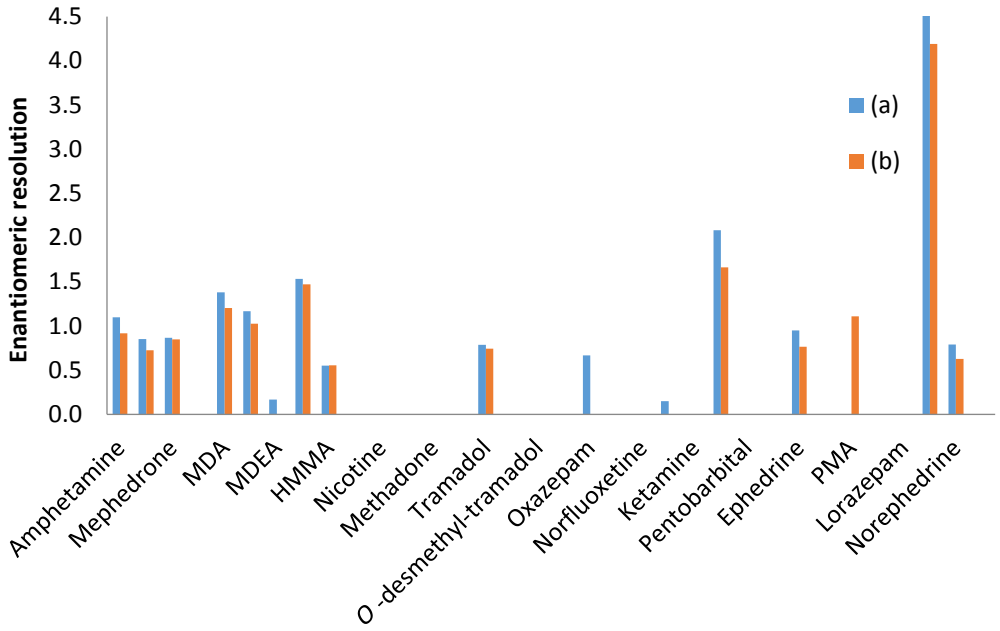



**Figure S2** CBH column - **e**nantiomeric resolution of studied analytes in a mobile phase containing isopropanol as organic modifier (mobile phase composition: (a) 1mM ammonium acetate/isopropanol 9: 1 and (b) 1mM ammonium acetate/isopropanol 9.5:0.5).

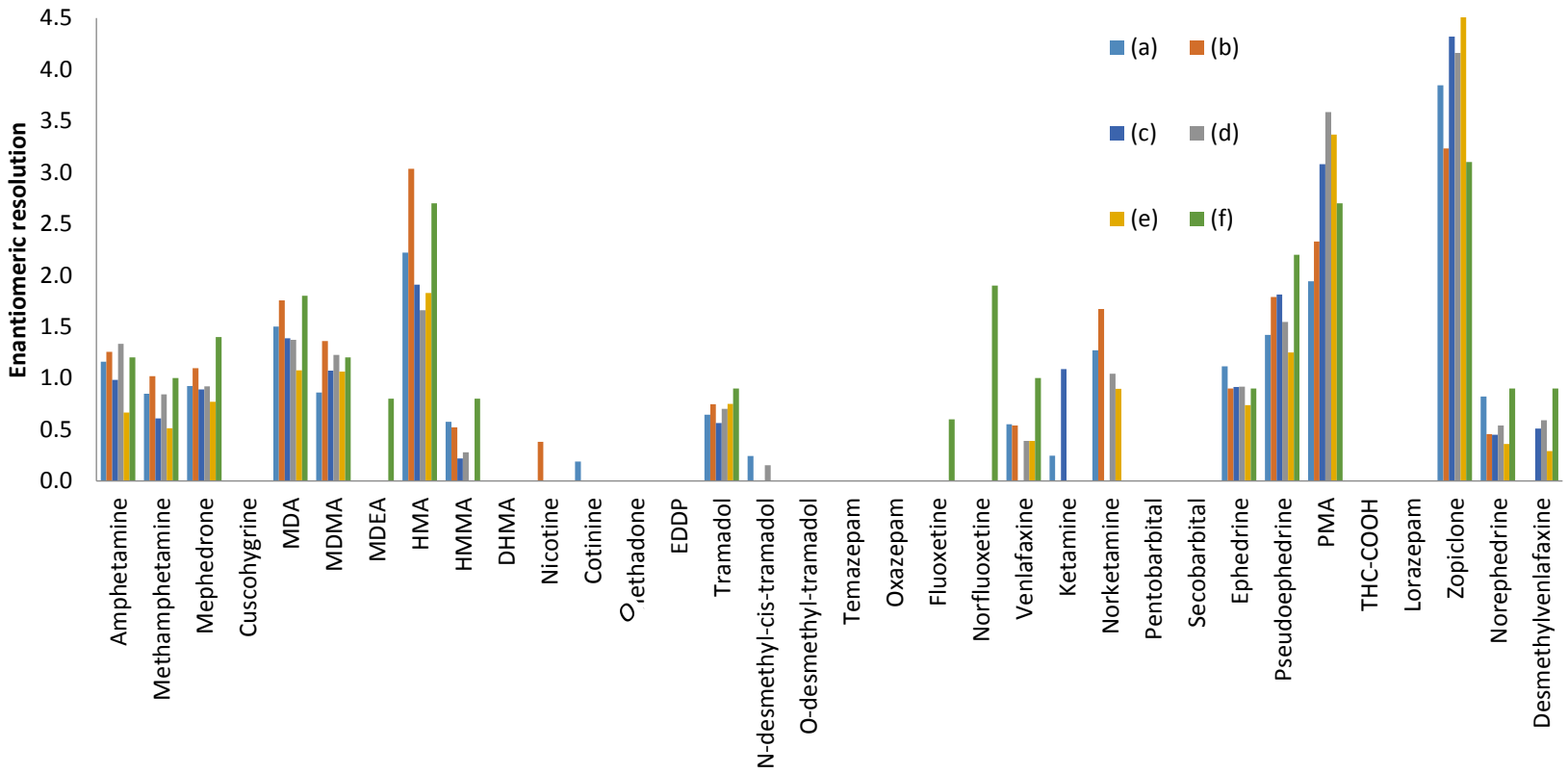
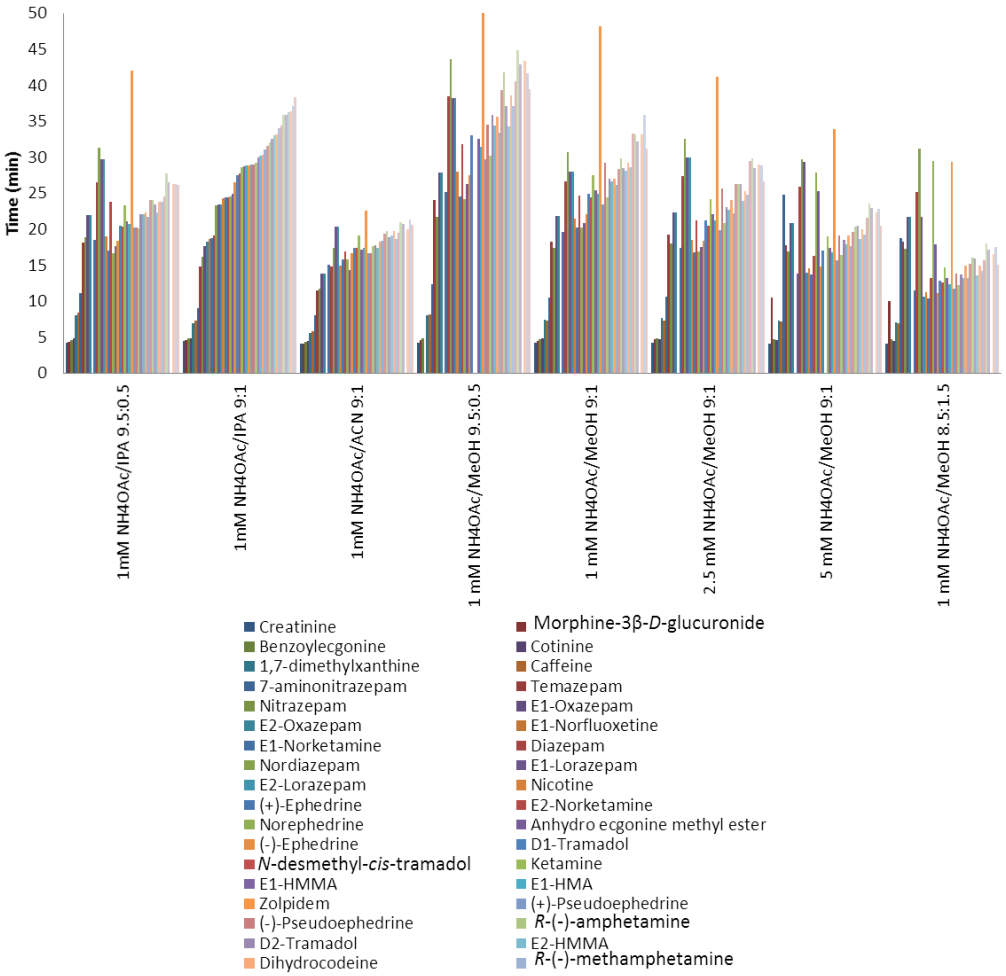



**Figure S3** CBH column - enantiomeric resolution of studied analytes in mobile phases containing: (a) 1 mM ammonium acetate/methanol 9.5:0.5, (b) 1 mM ammonium acetate/methanol 9:1, (c) 2.5 mM ammonium acetate/methanol 9:1, (d) 5 mM ammonium acetate /methanol 9:1, (e) 10 mM ammonium acetate /methanol 9:1 and (f) 1 mM ammonium acetate /methanol 8.5:1.5.


Time (min)
50
45
40
35
30
25
20
15
10
5
0
1mM NH4OAc/IPA 9.5:0.5
1mM NH4OAc/IPA 9:1
1mM NH4OAc/ACN 9:1
1 mM NH4OAc/MeOH 9.5:0.5
1 mM NH4OAc/MeOH 9:1
2.5 mM NH4OAc/MeOH 9:1
5 mM NH4OAc/MeOH 9:1
1 mM NH4OAc/MeOH 8.5:1.5
Creatinine
Benzoylecgonine
1,7-dimethylxanthine
7-aminonitrazepam
Nitrazepam
E2-Oxazepam
E1-Norketamine
Nordiazepam
E2-Lorazepam
(+)-Ephedrine
Norephedrine
(-)-Ephedrine
N-desmethyl-cis-tramadol
E1-HMMA
Zolpidem
(-)-Pseudoephedrine
D2-Tramadol
Dihydrocodeine
Morphine-3β-D-glucuronide
Cotinine
Caffeine
Temazepam
E1-Oxazepam
E1-Norfluoxetine
Diazepam
E1-Lorazepam
Nicotine
E2-Norketamine
Anhydro ecgonine methyl ester
D1-Tramadol
Ketamine
E1-HMA
(+)-Pseudoephedrine
R-(-)-amphetamine
E2-HMMA
R-(-)-methamphetamine

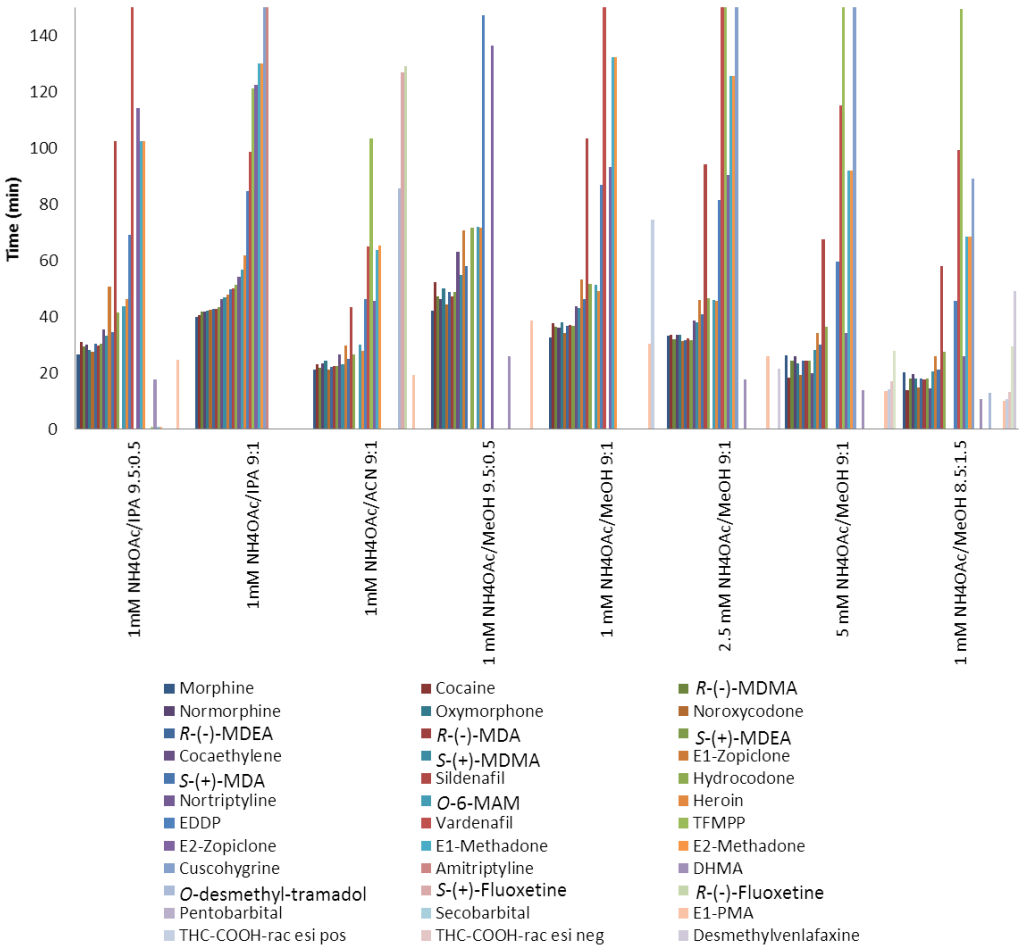



**Figure S4** CBH column – Impact of different percentages of modifiers on retention time of analytes ($NH_4OAc$: ammonium acetate, IPA: isopropanol, ACN: acetonitrile, MeOH: methanol).

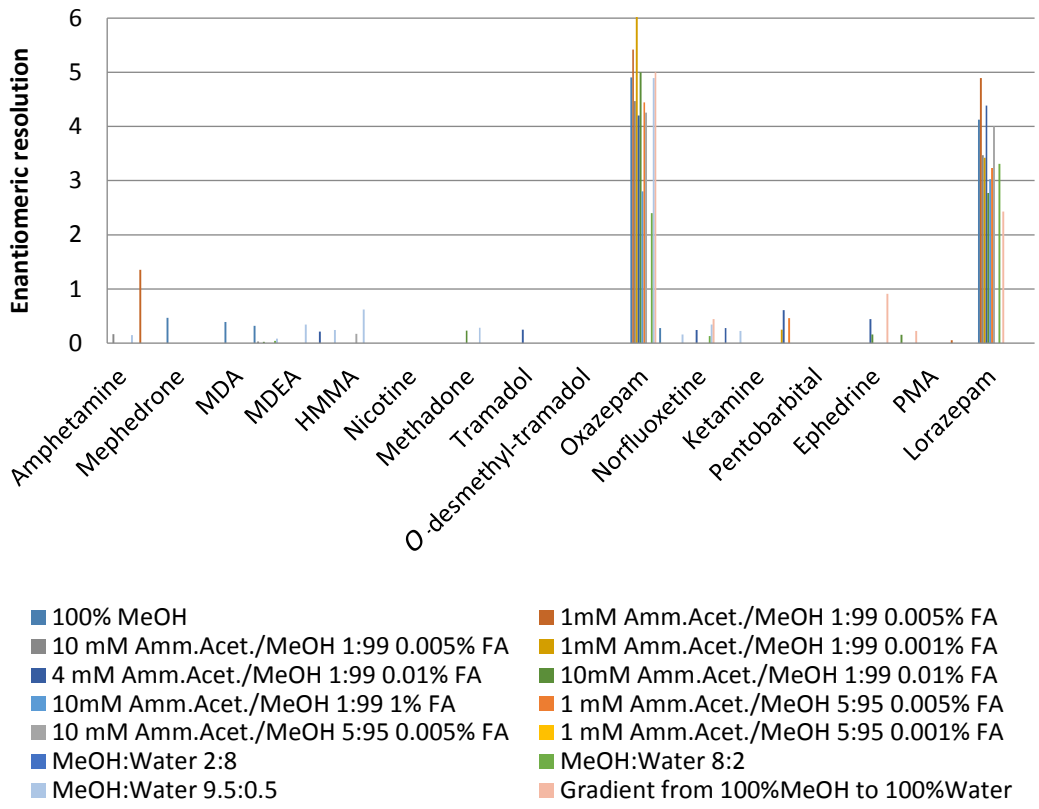



**Figure S5** Chirobiotic T column - overview of the separation for the targeted analytes (MeOH: methanol, Amm.Acet.: ammonium acetate, FA: formic acid).

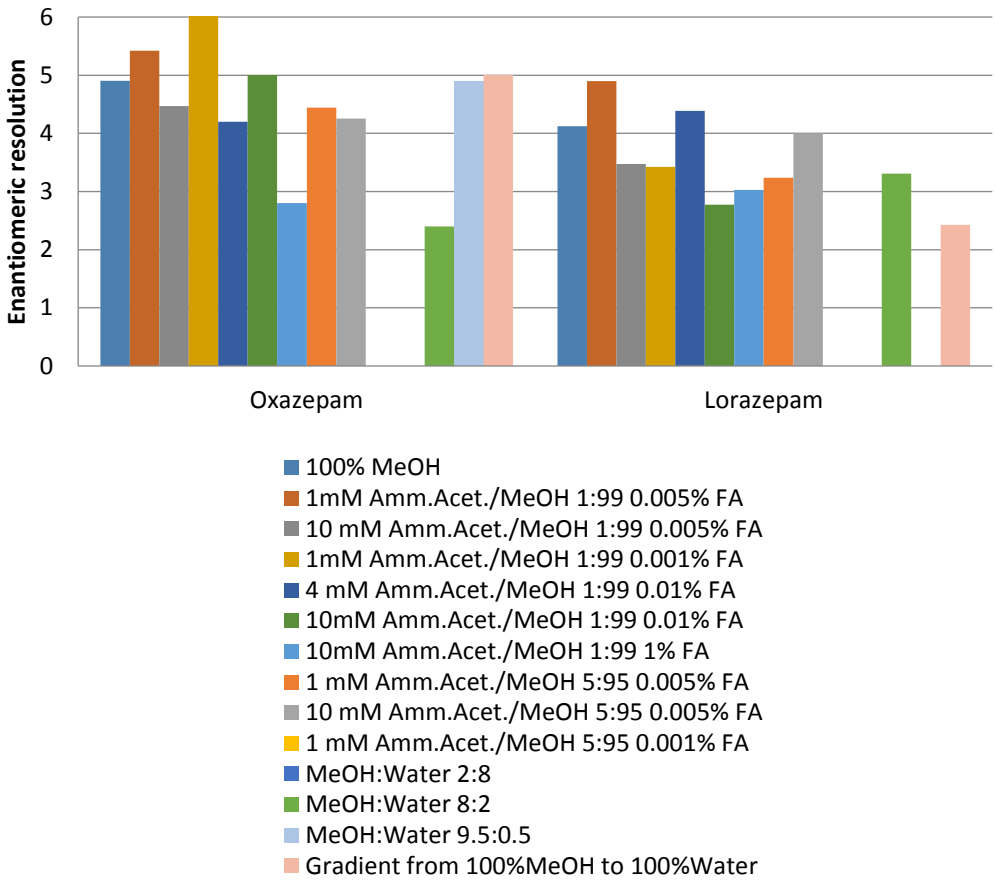



**Figure S6** Chirobiotic T column - separation of oxazepam and lorazepam (MeOH: methanol, Amm.Acet.: ammonium acetate, FA: formic acid.

# Appendix 2

The following supplementary data are contained in Appendix 2:

Figure S1 Synthesis of (±)-mephedrone (a, b) (modified from Schifano et al. (2010) [24]) and of *S-(-)*-mephedrone (c) (modified from Osorio-Olivares et al. (2003) [38]).

Figure S2 Proposed mephedrone metabolism in humans (modified from Pozo et al. (2014)).

Table S1 Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, a predicted using ACD/labs software (http://www.chemspider.com).

S1-Experimental settings and procedure for acetylation of rat urine sample.

Table S2 Experimental set up of the reactors used for (a) incubating wastewater and (b) pHLM.

Table S3 MRM transitions in chiral LC-TQD method.

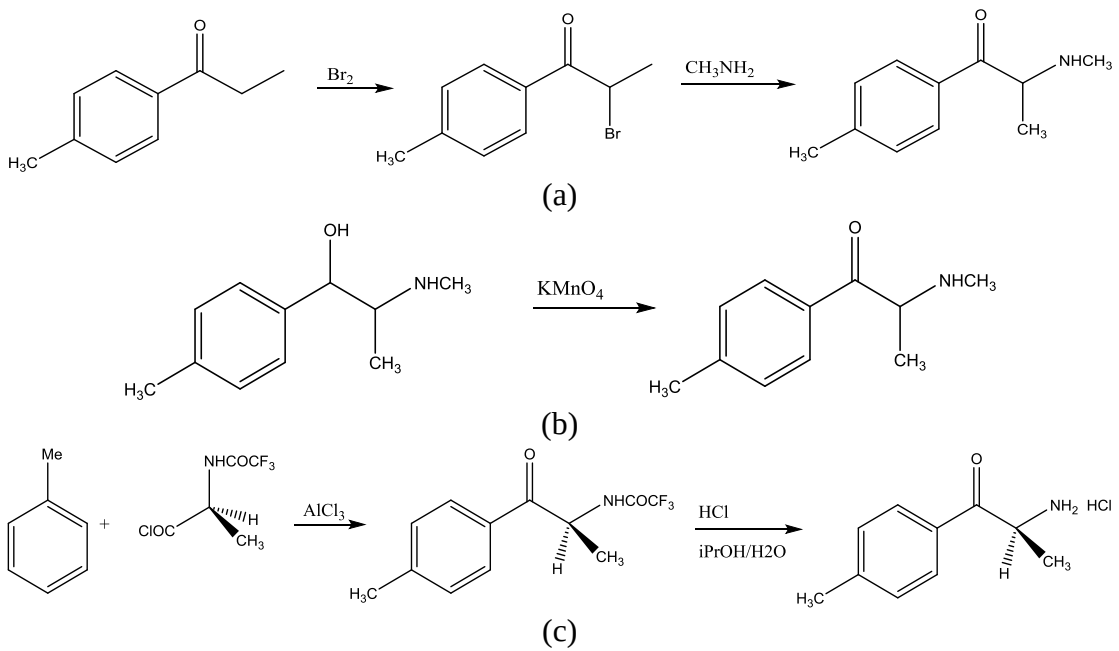



**Figure S1** Synthesis of (±)-mephedrone (a, b) (modified from Schifano et al. (2010) (Schifano *et al.*, 2011)) and of *S*-(-)-mephedrone (c) (modified from Osorio-Olivares et al. (2003) (Osorio-Olivares *et al.*, 2003))

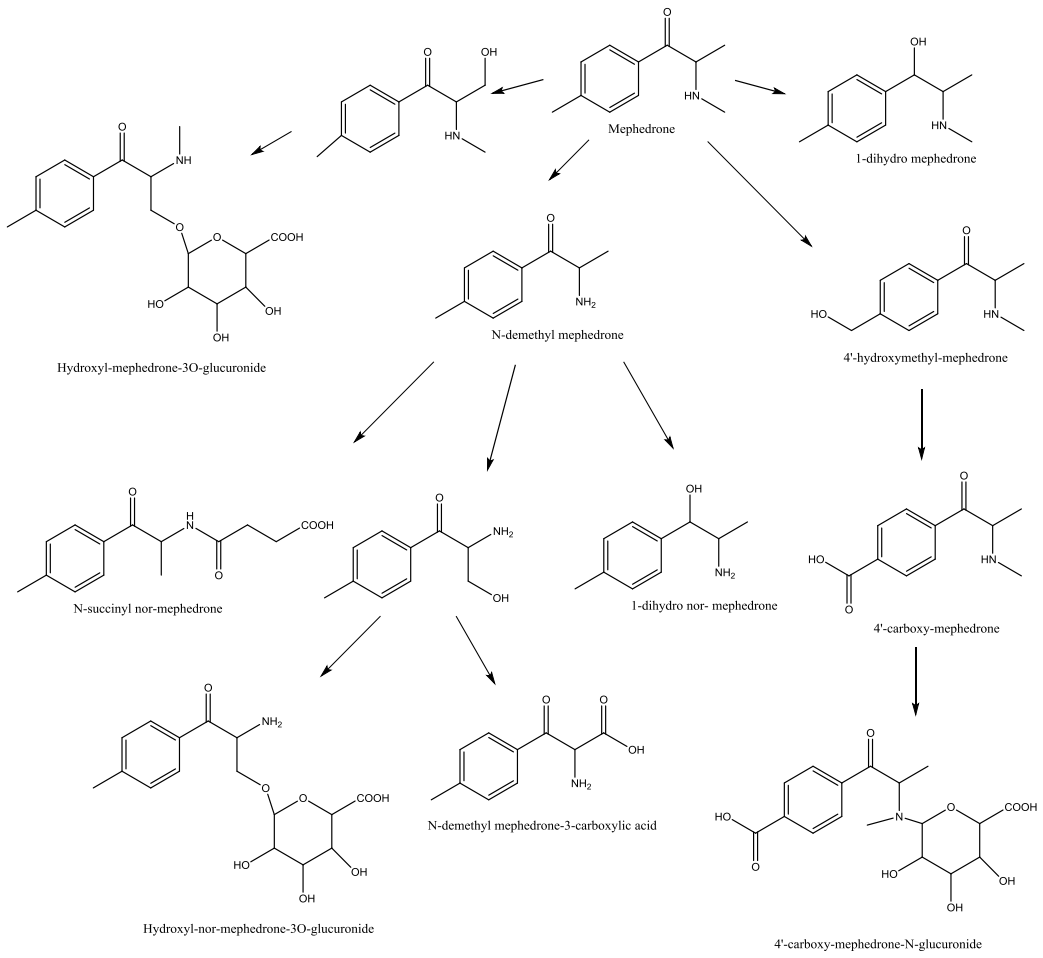



**Figure S2** Proposed mephedrone metabolism in humans (modified from Pozo et al. (2014)).

**Table S1** Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, [a] predicted using ACD/labs software (http://www.chemspider.com).

| Compound | CAS | Formula | MW | LogP | | LogD[a] | | Purity (%) | Supplier |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Exp. | Pred.[a] | pH 5.5 | pH 7.4 | | |
| (±)-Mephedrone | 189726-22-4 | $C_{11}H_{15}NO$ | 177.7 | - | 1.86±0.31 | -0.03 | 1.55 | 99.8 | Sigma-Aldrich (Cerilliant product) |
| (±)-Nor-mephedrone | 5941-17-9 | $C_{10}H_{13}NO$ | 163.4 | - | - | - | - | 98.0 | Cayman Chemical Company |
| (±)-Mephedrone-$D_3$ | 189972-79-9 | $C_{11}H_{12}NOD_3$ | 180.7 | | | | | 99.4 | Sigma-Aldrich |

**S1-Experimental settings and procedure for acetylation of rat urine sample.**

A blank and a positive rat urine sample underwent solid-phase extraction (SPE) with Isolute hydrophilic cation exchange (HCX) cartridges (130 mg, 3 mL, Biotage, Uppsala, Sweden). Cartridges were conditioned with 1mL methanol and 1mL of deionised water. 1 mL of rat urine sample spiked with 10 µL of 1 µg $mL^{-1}$ of mephedrone-$D_3$ was loaded onto the cartridge. The washing step was carried out with 1mL of deionised water, 1mL 0.01 M HCl followed by 1mL of deionised water. The neutral fraction was obtained after eluting the cartridges with 2 mL of methanol, and the basic fraction with 1mL methanol/$NH_3$ 33% mix 98: 2 v/v. The extracts were evaporated to dryness under nitrogen at 40°C and re-dissolved in 100 µL of methanol. 50 µL were transferred to other vials and dried under nitrogen flow at 40 °C. For the analysis in gas chromatography coupled to mass spectrometry (GC-MS), rat urine was acetylated with 100 µL of a mixture acetic acid/pyridine 3:2 through microwave irradiation for 5 minutes at 450 W. After evaporation, the sample was reconstituted in 50 µL of methanol and then injected in a splitless injection mode into a GC-MS system. The analysis was performed using a Hewlett Packard (HP, Agilent, Waldbronn, Germany) 5890 Series II gas chromatograph combined with an HP 5972A MSD mass spectrometer and an HP MS ChemStation (DOS series) with HP G1034C software version C03.00. The column was Thermo Scientific TG-1MS capillary (12 m × 0.2 mm I.D.), cross-linked methyl silicone, 330 nm film thickness. The following GC conditions were set: injection port temperature at 280 °C, helium as carrier gas, 1mL $min^{-1}$ as flow rate. The column temperature was programmed from 100 to 310 °C at 30 °C $min^{-1}$, initial time 2 min, final time 5 min. The MS conditions were as follows: electron ionization (EI) mode, 70 eV as ionization energy, ion source temperature at 220 °C and capillary direct interface heated at 280 °C. The acquisition was in full scan mode with m/z range from 50 to 550 uma.

**Table S2** Experimental set up of the reactors used for (a) incubating wastewater and (b) pHLM.

(a)

| | Wastewater reactors | | | |
|---|---|---|---|---|
| | Biotic | Abiotic | Clean | Control |
| Mephedrone (100 ng $mL^{-1}$) | X | X | X | |
| $NaN_3$ | | X | X | |
| Demineralised water | | | X | |
| Wastewater | X | X | | X |

(b)

| | Biological pHLM reactors | | |
|---|---|---|---|
| | Incubation | NoHLM | NoRegSys |
| Phosphate buffer pH 7.4 | X | X | X |
| Regenerating system (Reg Sys) | X | X | |
| Substrate solution | X | X | X |
| SOD | X | X | X |
| HLM | X | | X |

**Table S3** MRM transitions in chiral LC-TQD method

| **Compound** | **CV/CE[a]** | **MRM1 (quantification)** | **CV/CE[a]** | **MRM2 (confirmation)** | **CV/CE[a]** | **MRM3 (confirmation)** | **MRM1/MRM2 ratio ± SD** | **MRM1/MRM3 ratio ± SD** | **IS** |
|---|---|---|---|---|---|---|---|---|---|
| Mephedrone | 10/12 | 178.1 > 160.1 | 10/22 | 178.1 > 145.0 | 10/22 | 178.1 > 119.0 | 1.6 ± 0.2 | 8.5 ±2.1 | Mephedrone-D3 |
| Normephedrone | 10/20 | 164.0 > 131.0 | 10/32 | 164.0 > 91.0 | - | - | 6.6 ± 0.8 | - | Mephedrone-D3 |
| Mephedrone-D3 | 30/22 | 181.1 > 163.1 | - | - | - | - | - | - | - |

[a]CV, cone voltage (V); CE, collision energy (eV)

**Table S4** Method validation parameters (chiral LC-TQD) for mephedrone and normephedrone.

(a)

| Compound | $R_t$[a] (min) | Rel. $R_t$[a] | Sample diluent | | | | WWTP influent | |
|---|---|---|---|---|---|---|---|---|
| | | | Linear range (µg $L^{-1}$) | $R^2$ | $IDL_{S/N}$[b] (µg $L^{-1}$) | $IQL_{S/N}$[c] (µg $L^{-1}$) | MDL[d] (ng $L^{-1}$) | MQL[e] (ng $L^{-1}$) |
| *R*-(+)-Mephedrone | 16.5 ±0.4 | 0.3 | 0.25-500 | 0.9990 | 0.25 | 0.50 | 1.30 | 2.60 |
| *S*-(-)-Mephedrone | 21.0 ±0.5 | 0.2 | 0.25-500 | 0.9993 | 0.25 | 0.50 | 0.66 | 2.63 |
| *R*-(+)-Normephedrone | 44.2 ±0.8 | 6.5 | 0.25-500 | 0.9915 | 0.25 | 5.0 | 1.35 | 26.9 |
| *S*-(-)-Normephedrone | 68.8 ±1.4 | 6.7 | 0.25-500 | 0.9911 | 0.25 | 5.0 | 1.35 | 27.0 |

(b) SPE recovery % (n=3)

| | 25 ng/L | 250 ng/L | 2500 ng/L |
|---|---|---|---|
| *R*-(+)-Mephedrone | 109.1 ± 3.2 | 99.3 ± 4.8 | 80.7 ± 7.0 |
| *S*-(-)-Mephedrone | 99.0 ± 8.5 | 99.1 ± 4.3 | 87.2 ± 11.5 |
| *R*-(+)-Normephedrone | 72.8 ± 1.3 | 97.4 ± 9.2 | 108.5 ± 5.7 |
| *S*-(-)-Normephedrone | 79.4 ± 0.8 | 86.0 ± 2.0 | 113.2 ± 1.4 |

(c) Method precision; D represents day

| | Intra-day RSD% (n=4) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 ng $L^{-1}$ | 5 ng $L^{-1}$ | 5 ng $L^{-1}$ | 50 ng $L^{-1}$ | 50 ng $L^{-1}$ | 50 ng $L^{-1}$ | 500 ng $L^{-1}$ | 500 ng $L^{-1}$ | 500 ng $L^{-1}$ |
| | D 1 | D 2 | D 3 | D 1 | D 2 | D 3 | D 1 | D 2 | D 3 |
| *R*-(+)-Mephedrone | 9.8 | 13.7 | 14.1 | 3.6 | 6.8 | 14.6 | 3.7 | 10.0 | 5.6 |
| *S*-(-)-Mephedrone | 10.7 | 12.0 | 4.6 | 5.2 | 12.9 | 8.4 | 9.2 | 3.7 | 2.8 |
| *R*-(+)-Normephedrone | 14.1 | 20.8 | 13.3 | 2.8 | 13.3 | 8.3 | 1.5 | 5.5 | 12.8 |
| *S*-(-)-Normephedrone | 18.2 | 11.2 | 3.2 | 17.4 | 18.9 | 0.5 | 13.9 | 7.9 | 18.5 |

| | Inter-day RSD% (n=3) | | |
|---|---|---|---|
| | 5 ng $L^{-1}$ | 50 ng $L^{-1}$ | 500 ng $L^{-1}$ |
| *R*-(+)-Mephedrone | 12.5 | 8.3 | 6.4 |
| *S*-(-)-Mephedrone | 9.1 | 8.8 | 5.2 |
| *R*-(+)-Normephedrone | 10.3 | 8.1 | 6.6 |
| *S*-(-)-Normephedrone | 11.6 | 12.3 | 13.4 |

(d) Instrumental precision; D represents day

| | Intra-day RSD% (n=4) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 5 µg $L^{-1}$ | 5 µg $L^{-1}$ | 5 µg $L^{-1}$ | 50 µg $L^{-1}$ | 50 µg $L^{-1}$ | 50 µg $L^{-1}$ | 500 µg $L^{-1}$ | 500 µg $L^{-1}$ | 500 µg $L^{-1}$ |
| | D 1 | D 2 | D 3 | D 1 | D 2 | D 3 | D 1 | D 2 | D 3 |
| *R*-(+)-Mephedrone | 9.3 | 6.7 | 5.5 | 1.9 | 5.7 | 5.4 | 2.9 | 5.5 | 4.4 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *S*-(-)-Mephedrone | 3.5 | 6.7 | 1.1 | 3.6 | 2.5 | 2.7 | 9.3 | 4.3 | 2.2 |
| *R*-(+)-Normephedrone | 24.5 | 5.4 | 9.2 | 11.5 | 8.0 | 8.2 | 3.7 | 3.0 | 2.7 |
| *S*-(-)-Normephedrone | 27.5 | 12.4 | 1.1 | 5.6 | 4.5 | 6.6 | 4.3 | 2.1 | 4.0 |

(e)

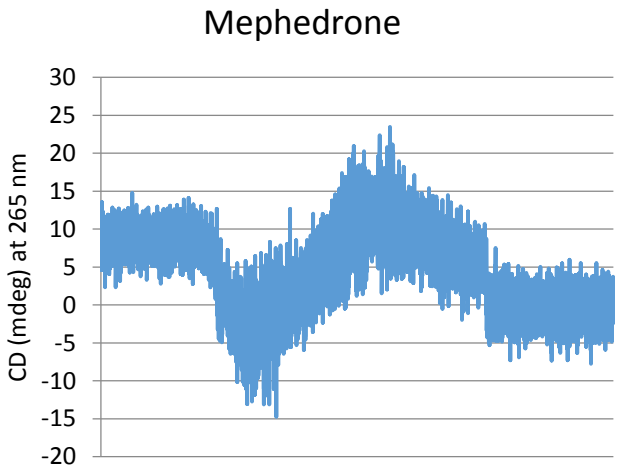
| | Inter-day RSD% (n=3) | | |
|---|---|---|---|
| | 5 µg $L^{-1}$ | 50 µg $L^{-1}$ | 500 µg $L^{-1}$ |
| *R*-(+)-Mephedrone | 7.1 | 4.3 | 4.3 |
| *S*-(-)-Mephedrone | 3.8 | 3.0 | 5.2 |
| *R*-(+)-Normephedrone | 8.5 | 9.2 | 3.1 |
| *S*-(-)-Normephedrone | 7.6 | 5.6 | 3.5 |

| | $R_s$[f] | EF[g] | | |
|---|---|---|---|---|
| | | 5 µg $L^{-1}$ | 50 µg $L^{-1}$ | 500 µg $L^{-1}$ |
| Mephedrone | 1.4 ±0.1 | 0.50±0.0 | 0.50±0.0 | 0.48±0.0 |
| Normephedrone | 4.0 ±0.4 | 0.51±0.0 | 0.50±0.0 | 0.50±0.0 |

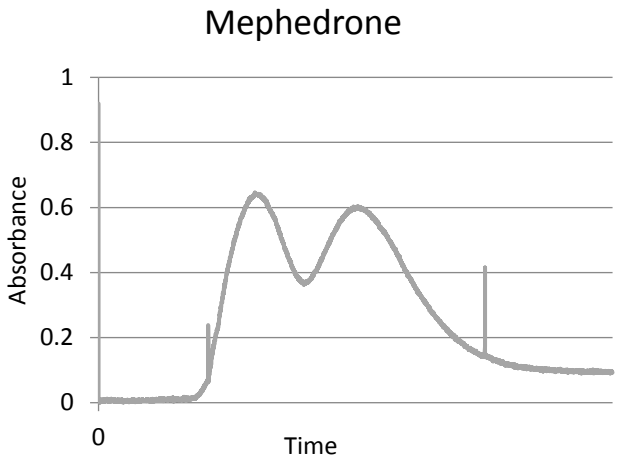
[a] Retention time; [b] Instrumental Limit of Detection (IDL). It was determined at a concentration value giving a signal-to-noise ratio (S/N) ≥ 3 for all the MRM transitions selected for cocaine; [c] Instrumental Limit of Quantification (IQL). It was determined at the minimum concentration value giving S/N ≥ 10 for all the MRM transitions; [d] Method Detection Limit (MDL); [e] Method Quantification Limit (MDL); [f] Enantiomeric resolution; [g] Enantiomeric fraction.

**Table S5** Operating conditions for the absorbance and CD spectra of (±)-mephedrone (4-MMC)

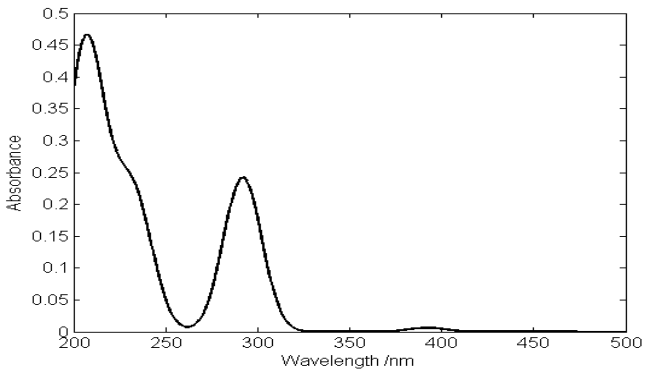
| | |
|---|---|
| Wavelength | 265 nm |
| Spectral bandwidth | 1 nm |
| Step size | 1 nm |
| Concentration | 170 µg mL-1 |
| Solvent | 1mM ammonium acetate/methanol 85:15 |
| Time | 1000 s |
| Points | 10000 |
| Samples | 4000 |
| Temperature (QNWPeltier) | 25.02 ºC |




(a)

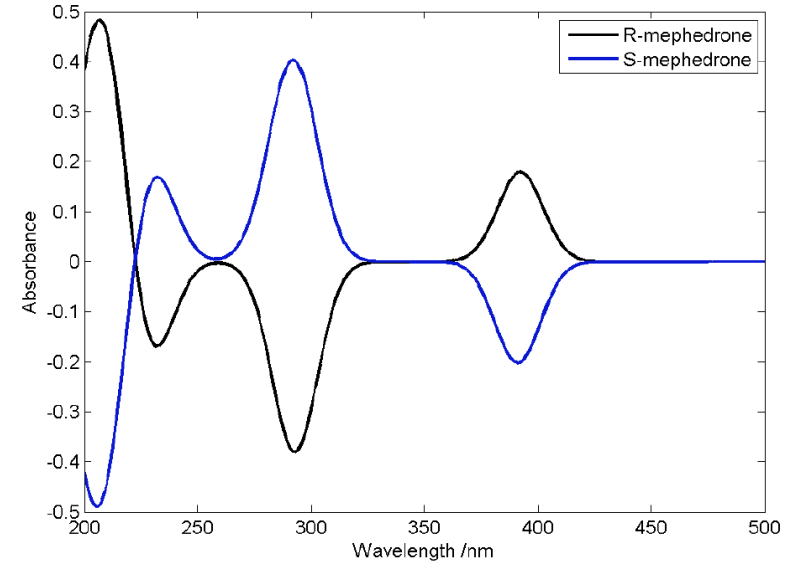



(b)

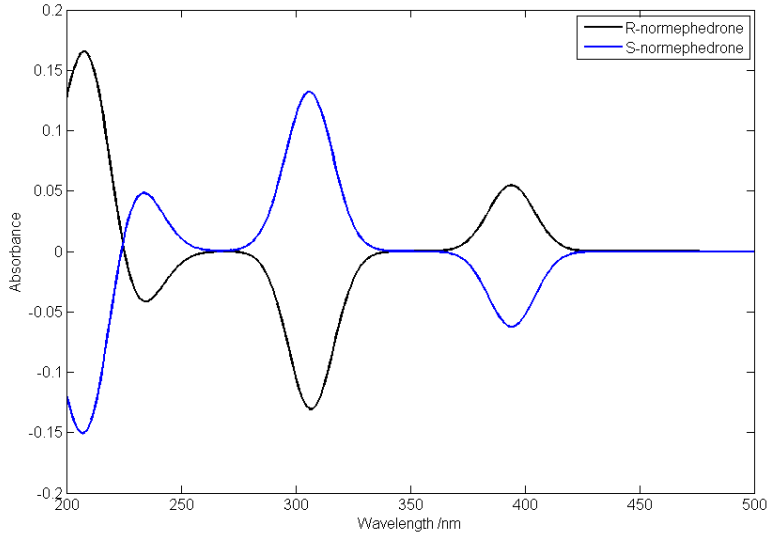



(c)

**Figure S3** CD and absorbance spectra of (±)-mephedrone (4-MMC) (a,b). UV spectrum of (±)-mephedrone from the computational study (c).




(a)




(b)

**Figure S4** Predicted CD spectra for (+)- and (-)-mephedrone (a) and for (+)- and (-)-normephedrone (b).

**S2-Statistical tests.**

Wastewater samples from 2014 sampling campaign.

F-Test Two-Sample for Variances

| | *EF* | *EF of standards from validation* |
|---|---|---|
| Mean | 0.563687 | 0.491920235 |
| Variance | 0.000983 | 0.000119448 |
| Observations | 7 | 7 |
| df | 6 | 6 |
| F | 8.232061 | |
| P(F<=f) one-tail | 0.010733 | |
| F Critical one-tail | 4.283866 | |

Data:

| UK 2014 | Validation |
|---|---|
| EF | EF of standards |
| 0.59 | 0.49 |
| 0.57 | 0.51 |
| 0.51 | 0.49 |
| 0.55 | 0.50 |
| 0.57 | 0.50 |
| 0.61 | 0.48 |
| 0.57 | 0.47 |

$\alpha$=0.05

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards from validation |
| Mean | 0.5636866 | 0.491920235 |
| Variance | 0.000983302 | 0.000119448 |
| Observations | 7 | 7 |
| Hypothesized Mean Difference | 0 | |
| df | 7 | |
| t Stat | 5.71783294 | |
| P(T<=t) one-tail | 0.000360966 | |
| t Critical one-tail | 1.894578605 | |
| P(T<=t) two-tail | 0.000721932 | |
| t Critical two-tail | 2.364624252 | |

$\alpha$=0.001

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| t Stat | 5.71783294 | |
| P(T<=t) one-tail | 0.000360966 | |
| t Critical one-tail | 4.785289629 | |
| P(T<=t) two-tail | 0.000721932 | |
| t Critical two-tail | 5.407882521 | |

Wastewater samples from 2014 sampling campaign (without Wednesday).

Data:

| UK 2014 | Validation |
|---|---|
| EF | EF of standards |
| 0.59 | 0.49 |
| 0.57 | 0.51 |
| - | 0.49 |
| 0.55 | 0.50 |
| 0.57 | 0.50 |
| 0.61 | 0.48 |
| 0.57 | 0.47 |

α=0.05

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| Mean | 0.573260904 | 0.491920235 |
| Variance | 0.000409957 | 0.000119448 |
| Observations | 6 | 7 |
| Hypothesized Mean Difference | 0 | |
| df | 7 | |
| t Stat | 8.802454788 | |
| P(T<=t) one-tail | 0.00002464 | |
| t Critical one-tail | 1.894578605 | |
| P(T<=t) two-tail | 4.92701E-05 | |
| t Critical two-tail | 2.364624252 | |

α=0.001

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| t Stat | 8.802454788 | |
| P(T<=t) one-tail | 0.00002464 | |
| t Critical one-tail | 4.785289629 | |
| P(T<=t) two-tail | 4.92701E-05 | |
| t Critical two-tail | 5.407882521 | |

## Wastewater samples from 2015 sampling campaign.

F-Test Two-Sample for Variances

| | *EF* | *EF of standards from validation* |
|---|---|---|
| Mean | 0.536691 | 0.49192 |
| Variance | 0.002805 | 0.000119 |
| Observations | 7 | 7 |
| df | 6 | 6 |
| F | 23.48412 | |
| P(F<=f) one-tail | 0.00064 | |
| F Critical one-tail | 4.283866 | |

Data:

| UK 2015 | Validation |
|---|---|
| EF | EF of standards |
| 0.47 | 0.49 |

| | |
|---|---|
| 0.53 | 0.51 |
| 0.50 | 0.49 |
| 0.53 | 0.50 |
| 0.61 | 0.50 |
| 0.50 | 0.48 |
| 0.60 | 0.47 |

α=0.05

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| Mean | 0.536690782 | 0.491920235 |
| Variance | 0.002805128 | 0.000119448 |
| Observations | 7 | 7 |
| Hypothesized Mean Difference | 0 | |
| df | 7 | |
| t Stat | 2.19033233 | |
| P(T<=t) one-tail | 0.032323525 | |
| t Critical one-tail | 1.894578605 | |
| P(T<=t) two-tail | 0.06464705 | |
| t Critical two-tail | 2.364624252 | |

α=0.001

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| t Stat | 2.19033233 | |
| P(T<=t) one-tail | 0.032323525 | |
| t Critical one-tail | 4.785289629 | |
| P(T<=t) two-tail | 0.06464705 | |
| t Critical two-tail | 5.407882521 | |

Wastewater samples from 2015 sampling campaign (without Monday).

Data:

| UK 2015 | Validation |
|---|---|
| EF | EF of standards |
| - | 0.49 |
| 0.53 | 0.51 |
| 0.50 | 0.49 |
| 0.53 | 0.50 |
| 0.61 | 0.50 |
| 0.50 | 0.48 |
| 0.60 | 0.47 |

α=0.05

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| Mean | 0.548157004 | 0.491920235 |
| Variance | 0.002261769 | 0.000119448 |
| Observations | 6 | 7 |
| Hypothesized Mean Difference | 0 | |

| | | |
|---|---|---|
| df | 5 | |
| t Stat | 2.833075718 | |
| P(T<=t) one-tail | 0.018271203 | |
| t Critical one-tail | 2.015048373 | |
| P(T<=t) two-tail | 0.036542405 | |
| t Critical two-tail | 2.570581836 | |

α=0.001

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| t Stat | 2.833075718 | |
| P(T<=t) one-tail | 0.018271203 | |
| t Critical one-tail | 5.893429531 | |
| P(T<=t) two-tail | 0.036542405 | |
| t Critical two-tail | 6.868826626 | |

## Comparison between wastewater samples in 2014-15 sampling campaigns.

Data:

| UK 2014 | UK 2015 |
|---|---|
| EF | EF |
| 0.59 | 0.47 |
| 0.57 | 0.53 |
| - | 0.50 |
| 0.55 | 0.53 |
| 0.57 | 0.61 |
| 0.61 | 0.50 |
| 0.57 | 0.60 |

α=0.05

| t-Test: Paired Two Sample for Means | | |
|---|---|---|
| | EF | EF |
| Mean | 0.5636866 | 0.536690782 |
| Variance | 0.000983302 | 0.002805128 |
| Observations | 7 | 7 |
| Pearson Correlation | -0.040609127 | |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | 1.140299108 | |
| P(T<=t) one-tail | 0.148812821 | |
| t Critical one-tail | 1.943180281 | |
| P(T<=t) two-tail | 0.297625643 | |
| t Critical two-tail | 2.446911851 | |

## Illegal street mephedrone samples.

F-Test Two-Sample for Variances

| | *EF of standards from validation* | *EF* |
|---|---|---|
| Mean | 0.491920235 | 0.500885962 |
| Variance | 0.000119448 | 0.0000718 |
| Observations | 7 | 8 |
| df | 6 | 7 |

| | |
|---|---|
| F | 1.664004881 |
| P(F<=f) one-tail | 0.259739457 |
| F Critical one-tail | 3.865968853 |

Data:

| Street mephedrone samples | Validation |
|---|---|
| EF | EF of standards |
| 0.51 | 0.49 |
| 0.49 | 0.51 |
| 0.51 | 0.49 |
| 0.49 | 0.50 |
| 0.50 | 0.50 |
| 0.50 | 0.48 |
| 0.50 | 0.47 |
| 0.50 | - |

α=0.05

| t-Test: Two-Sample Assuming Equal Variances | | |
|---|---|---|
| | EF | EF of standards |
| Mean | 0.500885962 | 0.491920235 |
| Variance | 7.17834E-05 | 0.000119448 |
| Observations | 8 | 7 |
| Pooled Variance | 9.37824E-05 | |
| Hypothesized Mean Difference | 0 | |
| df | 13 | |
| t Stat | 1.788847491 | |
| P(T<=t) one-tail | 0.048476721 | |
| t Critical one-tail | 1.770933396 | |
| P(T<=t) two-tail | 0.096953442 | |

## Pooled human urine samples.

F-Test Two-Sample for Variances

| | *EF* | *EF of standards from validation* |
|---|---|---|
| Mean | 0.587123641 | 0.491920235 |
| Variance | 0.002536336 | 0.000119448 |
| Observations | 7 | 7 |
| df | 6 | 6 |
| F | 21.23383177 | |
| P(F<=f) one-tail | 0.000849547 | |
| F Critical one-tail | 4.283865714 | |

Data:

| Pooled human urine samples | Validation |
|---|---|
| EF | EF of standards |
| 0.50 | 0.49 |
| 0.57 | 0.51 |
| 0.59 | 0.49 |
| 0.61 | 0.50 |

| | |
|---|---|
| 0.65 | 0.50 |
| 0.62 | 0.48 |
| 0.56 | 0.47 |

α=0.05

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| Mean | 0.587124 | 0.49192 |
| Variance | 0.002536 | 0.000119 |
| Observations | 7 | 7 |
| Hypothesized Mean Difference | 0 | |
| df | 7 | |
| t Stat | 4.887707 | |
| P(T<=t) one-tail | 0.000889 | |
| t Critical one-tail | 1.894579 | |
| P(T<=t) two-tail | 0.001778 | |
| t Critical two-tail | 2.364624 | |

α=0.001

| t-Test: Two-Sample Assuming Unequal Variances | | |
|---|---|---|
| | EF | EF of standards |
| t Stat | 4.887707 | |
| P(T<=t) one-tail | 0.000889 | |
| t Critical one-tail | 4.78529 | |
| P(T<=t) two-tail | 0.001778 | |
| t Critical two-tail | 5.407883 | |

**Table S6** Mephedrone concentrations and population-normalised mass loads in wastewater samples during one week monitoring campaign in 2014 and in 2015 in the UK.

| **2014** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Day** | **Flow [$m^3$ $day^{-1}$]** | **PE[a]** | ***R*-(+)-Mephedrone** | | ***S*-(-)-Mephedrone** | | **(±)-Mephedrone** | |
| | | | Concentration [ng $L^{-1}$] | Population-normalised mass loads [mg 1000 $people^{-1}day^{-1}$] | Concentration [ng $L^{-1}$] | Population-normalised mass loads [mg 1000 $people^{-1}day^{-1}$] | Concentration [ng $L^{-1}$] | Population-normalised mass loads [mg 1000 $people^{-1}day^{-1}$] |
| Monday | 199012 | 886650 | 42 ± 7 | 9.3 | 29 ± 2 | 6.5 | 70.5 | 15.8 |
| Tuesday | 216049 | 886650 | 18 ± 6 | 4.3 | 14 ± 5 | 3.4 | 31.8 | 7.7 |
| Wednesday | 214229 | 886650 | 32 ± 10 | 7.7 | 28 ± 3 | 6.6 | 59.3 | 14.3 |
| Thursday | 208782 | 886650 | 18 ± 3 | 4.2 | 14 ± 6 | 3.4 | 32.3 | 7.6 |
| Friday | 208644 | 886650 | 22 ± 7 | 5.1 | 18 ± 5 | 4.3 | 40.0 | 9.4 |
| Saturday | 204287 | 886650 | 67 ± 15 | 15.4 | 47 ± 6 | 10.8 | 114.0 | 26.3 |
| Sunday | 198221 | 886650 | 53 ± 11 | 11.8 | 44 ± 5 | 9.8 | 96.5 | 21.6 |
| Average | | | | 8.3 | | 6.4 | | 14.7 |
| SD | | | | 4.2 | | 3.0 | | 7.2 |
| CV | | | | 0.5 | | 0.5 | | 0.5 |
| **2015** | | | | | | | | |
| **Day** | **Flow [$m^3$ $day^{-1}$]** | **PE[a]** | ***R*-(+)-Mephedrone** | | ***S*-(-)-Mephedrone** | | **(±)-Mephedrone** | |
| | | | Concentration [ng $L^{-1}$] | Population-normalised mass loads [mg 1000 $people^{-1}day^{-1}$] | Concentration [ng $L^{-1}$] | Population-normalised mass loads [mg 1000 $people^{-1}day^{-1}$] | Concentration [ng $L^{-1}$] | Population-normalised mass loads [mg 1000 $people^{-1}day^{-1}$] |
| Monday | 197493 | 886650 | 67± 5 | 14.9 | 72 ±7 | 16.1 | 140 | 31.1 |
| Tuesday | 204491 | 886650 | 37 ± 6 | 8.5 | 27 ± 2 | 6.3 | 65 | 14.9 |
| Wednesday | 198950 | 886650 | 38 ± 3 | 8.5 | 38 ± 3 | 8.5 | 76 | 17.1 |
| Thursday | 197523 | 886650 | 37 ± 5 | 8.2 | 33 ± 2 | 7.4 | 70 | 15.6 |
| Friday | 252682 | 886650 | 45 ± 2 | 12.7 | 28 ± 1 | 8.1 | 73 | 20.8 |

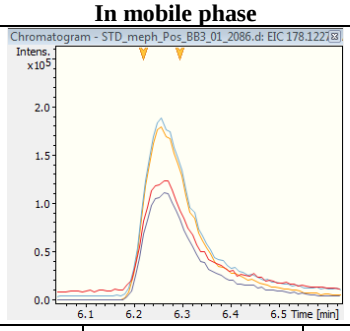
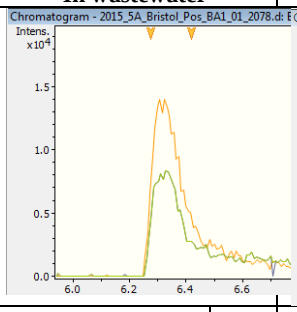
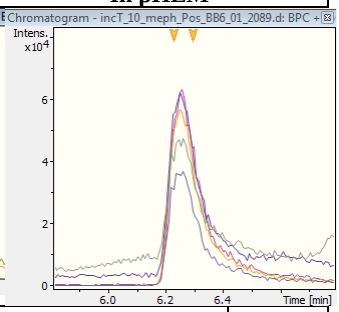
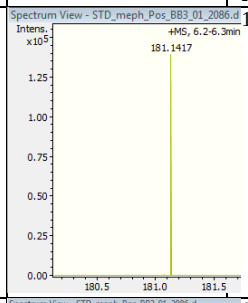
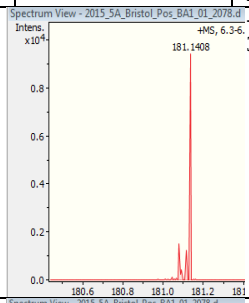
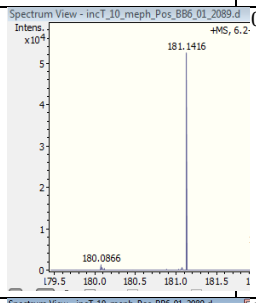
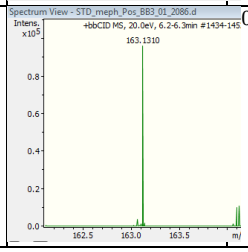
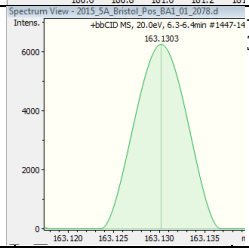
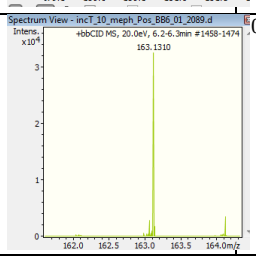
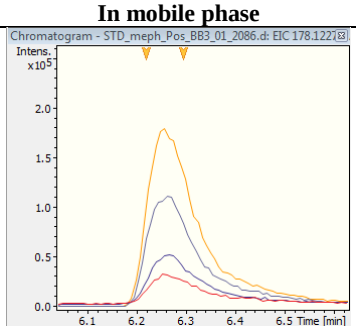
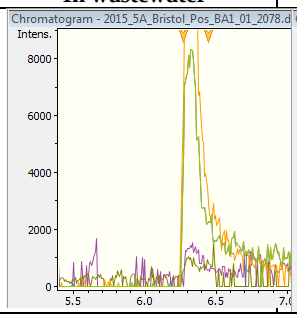
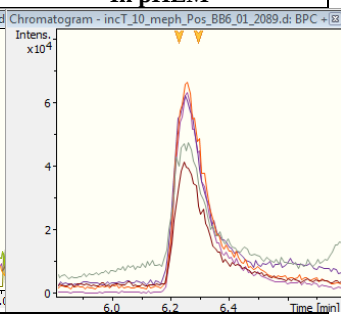
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Saturday | 220687 | 886650 | 106 ± 5 | 26.3 | 86 ± 4 | 21.4 | 192 | 47.7 |
| Sunday | 193194 | 886650 | 90 ± 6 | 19.6 | 58 ± 2 | 12.5 | 148 | 32.1 |
| Average | | | | 14.1 | | 11.5 | | 25.6 |
| SD | | | | 6.8 | | 5.6 | | 12.0 |
| CV | | | | 0.5 | | 0.5 | | 0.5 |

[a] Population Equivalent

**Table S7** Non-targeted analysis by LC Q-TOF: mephedrone metabolites predicted in wastewater and in pHLM by using MetID software (*Theor* means theoretical and *Exp.* experimental).

| **Metabolite** | **Formula** | **Ionization mode** | **Rt** | **Precursor ion** | | |
|---|---|---|---|---|---|---|
| | | | | **Theor. mass ($[M+H]^+$ or $[M-H]^{l-}$)** | **Exp. mass ($[M+H]^+$or $[M-H]^{l-}$)** | **Mass Error (ppm)** |
| ***Wastewater*** | | | | | | |
| Dihydro-mephedrone | C11H17NO | ESI + | 5.50 | 180.1383 | 180.1384 | 0.55 |
| Hydroxy-tolyl-normephedrone | C10H13NO2 | ESI - | 6.70 | 178.0874 | 178.0871 | -1.68 |
| 4'-carboxy mephedrone | C11H13NO3 | ESI - | 5.85 | 206.0823 | 206.0822 | 0.48 |
| 4'-carboxy normephedrone | C10H11NO3 | ESI - | 6.92 | 192.067 | 192.066 | 0.52 |
| *N*-sulfo-normephedrone | C10H13NO4S | ESI + | 7.15 | 244.0640 | 244.0638 | 0.82 |
| ***pHLM*** | | | | | | |
| Normephedrone | C10H13NO | ESI + | 6.18 | 164.1070 | 164.1064 | 3.65 |
| 4'-carboxy-normephedrone | C10H11NO3 | ESI - | 6.90 | 194.0812 | 194.0820 | 4.12 |

**Table S8** Target screening analysis in wastewater and in pHLM by using LC-QTOF.

| STANDARD MEPHEDRONE-D3 | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **In mobile phase** | | | **In wastewater** | | **In pHLM** | |
| **Chromatogram** |  | | |  | |  | |
| | **Theor. mass [M+H]$^+$** | **Experimental mass [M+H]$^+$** | **Mass Error (ppm)** | **Experimental mass [M+H]$^+$** | **Mass Error (ppm)** | **Experimental mass [M+H]$^+$** | **Mass Error (ppm)** |
| **Precursor ion** | 181.1415 |  | 1.10 |  | -3.86 |  | 0.55 |
| **Fragment ion** | 163.1309 |  | 0.61 |  | -3.68 |  | 0.61 |
| **MEPHEDRONE** | | | | | | | |
| | **In mobile phase** | | | **In wastewater** | | **In pHLM** | |
| **Chromatogram** |  | | |  | |  | |
| | **Theor. mass [M+H]$^+$** | **Experimental mass [M+H]$^+$** | **Mass Error (ppm)** | **Experimental mass [M+H]$^+$** | **Mass Error (ppm)** | **Experimental mass [M+H]$^+$** | **Mass Error (ppm)** |

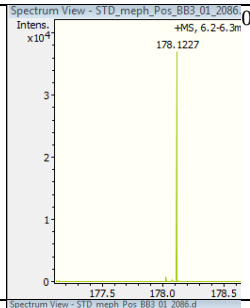
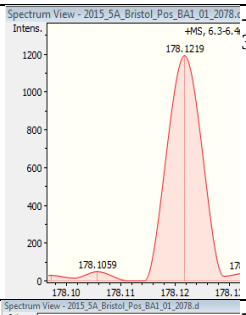
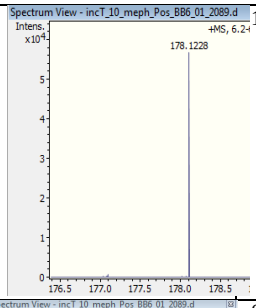
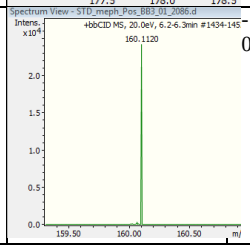
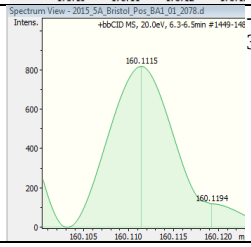
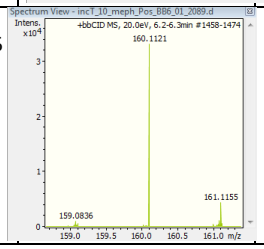
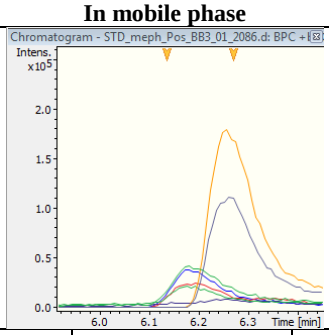
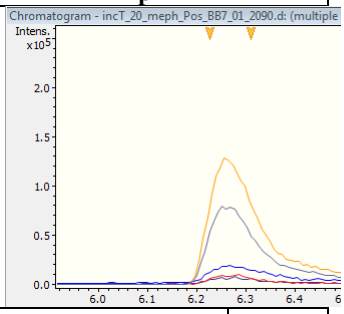
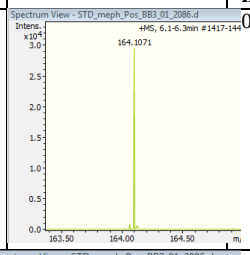
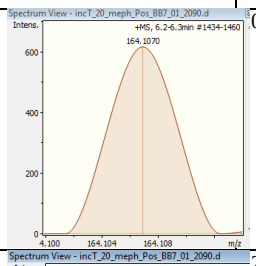
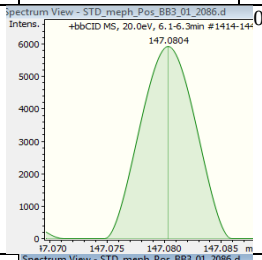
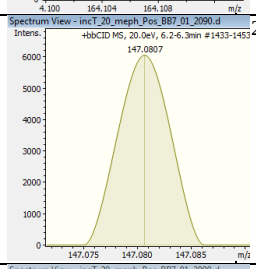
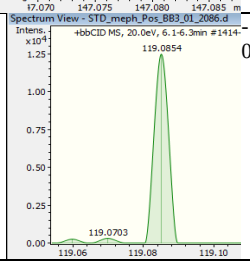
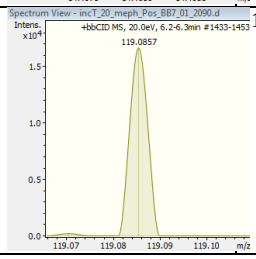
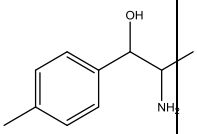
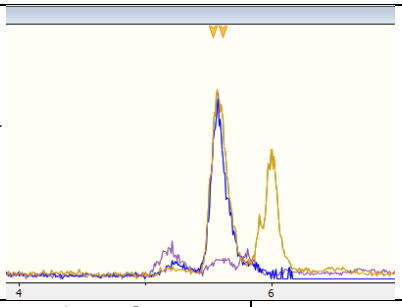
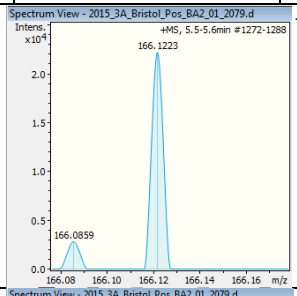
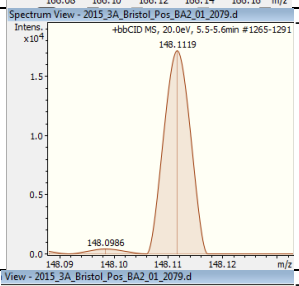
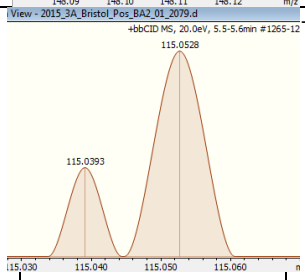
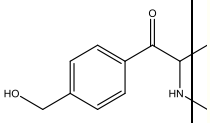
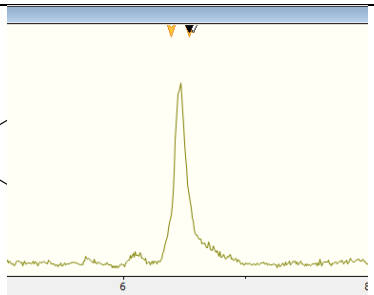
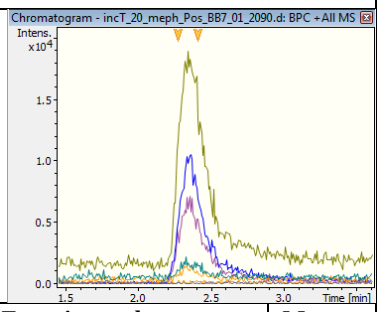
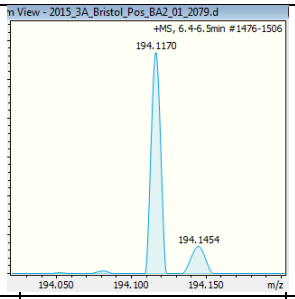
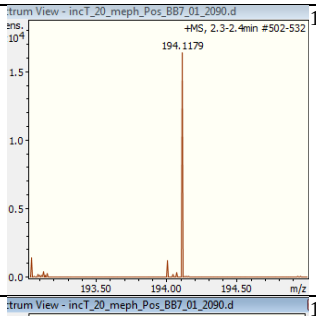
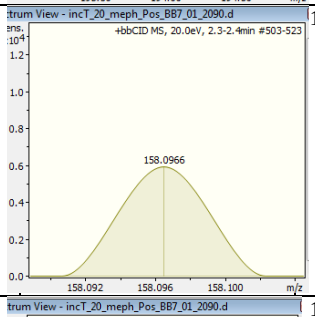
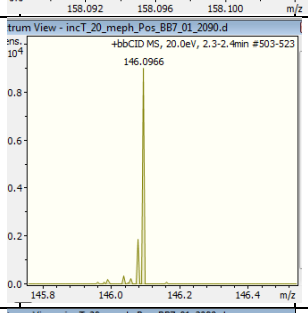
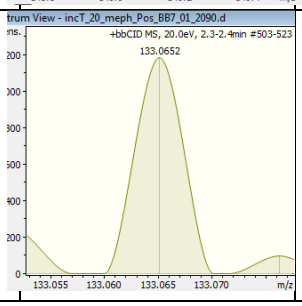
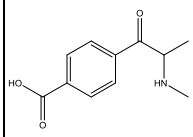
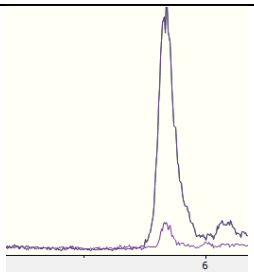
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Precursor ion** | 178.1226 |  | 0.56 | - |  | 3.93 |  | 1.12 |
| **Fragment ion** | 160.1121 |  | 0.62 | - |  | 3.75 |  | 0 |
| **NORMEPHEDRONE** | | | | | | | | |
| | | **In mobile phase** | | | **In wastewater** | | **In pHLM** | |
| **Chromatogram** | |  | | - | | |  | |
| | **Theor. mass [M+H]+** | **Experimental mass [M+H]+** | **Mass Error (ppm)** | **Experimental mass [M+H]+** | | **Mass Error (ppm)** | **Experimental mass [M+H]+** | **Mass Error (ppm)** |
| **Precursor ion** | 164.1070 |  | 0.61 | - | | n.d. |  | 0 |
| **Fragment ion** | 147.0804 |  | 0 | - | | n.d. |  | 2.04 |
| **Fragment ion** | 119.0855 |  | 0.84 | - | | n.d. |  | 1.68 |

**Table S9** Untarget screening analysis in wastewater and in pHLM by using LC-QTOF.

| **1-DIHYDRO NORMEPHEDRONE** | | | | | |
|---|---|---|---|---|---|
| | **Structure** | **In wastewater** | | **In pHLM** | |
| **Chromatogram** |  |  | | - | |
| | **Theor. mass $[M+H]^+$** | **Experimental mass $[M+H]^+$** | **Mass Error (ppm)** | **Experimental mass $[M+H]^+$** | **Mass Error (ppm)** |
| **Precursor ion** | 166.1226 |  | -1.80 | - | - |
| **Fragment ion** | 148.1121 |  | -1.35 | - | - |
| **Fragment ion** | 115.0542 |  | -8.70 | - | - |
| **4'-HYDROXYMETHYL-MEPHEDRONE** | | | | | |
| | **Structure** | **In wastewater** | | **In pHLM** | |
| **Chromatogram** |  |  | |  | |
| | **Theor. mass $[M+H]^+$** | **Experimental mass $[M+H]^+$** | **Mass Error (ppm)** | **Experimental mass $[M+H]^+$** | **Mass Error (ppm)** |

| | | | | | |
|---|---|---|---|---|---|
| **Precursor ion** | 194.1176 |  | -3.09 |  | 1.54 |
| **Fragment ion** | 158.0964 | | - |  | 1.26 |
| **Fragment ion** | 146.0964 | | - |  | 1.37 |
| **Fragment ion** | 133.0648 | | |  | 3.00 |

| **4'-CARBOXY-MEPHEDRONE** | | | | | |
|---|---|---|---|---|---|
| | **Structure** | **In wastewater** | | **In pHLM** | |
| **Chromatogram** |  |  | | - | |
| | **Theor. mass $[M+H]^+$** | **Experimental mass $[M+H]^+$** | **Mass Error (ppm)** | **Experimental mass $[M+H]^+$** | **Mass Error (ppm)** |

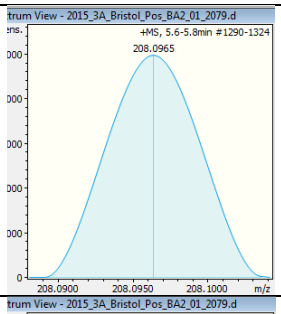
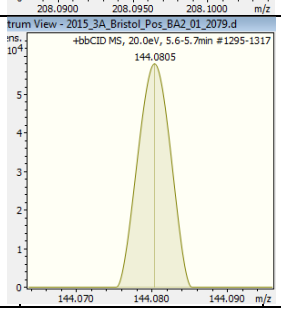
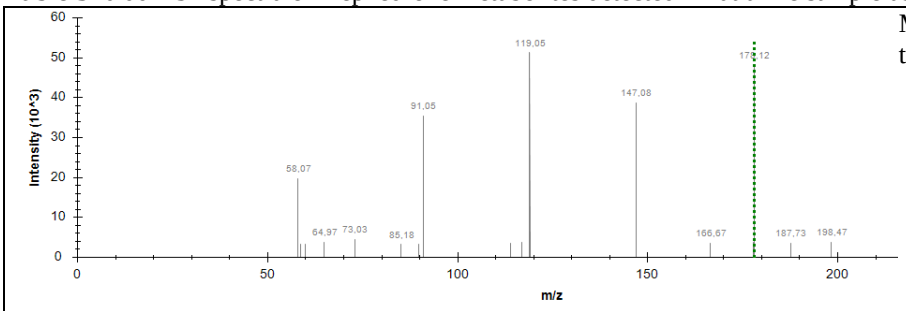
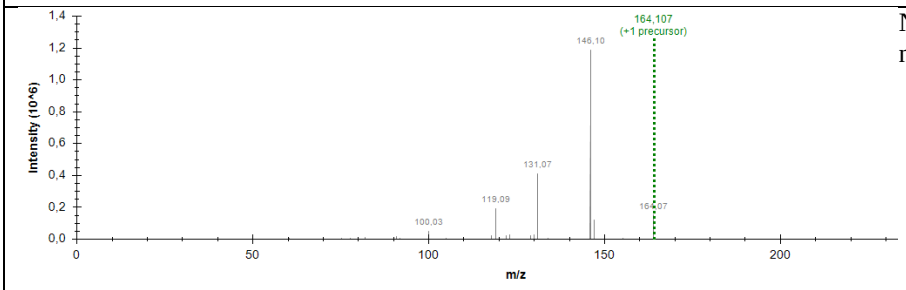
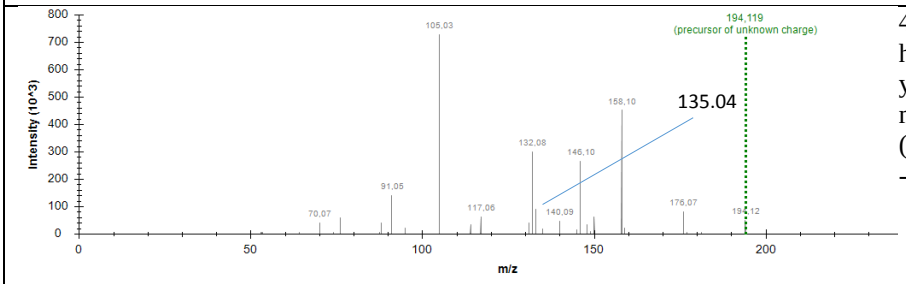
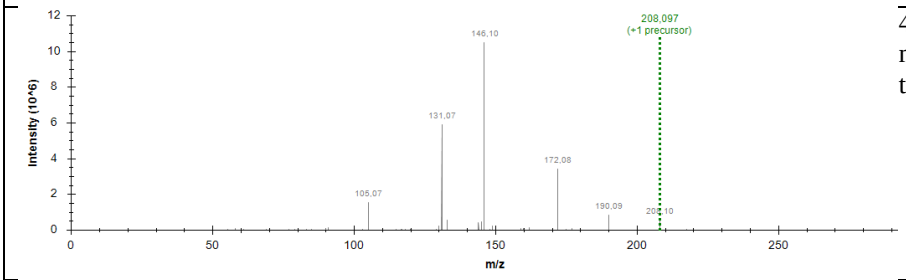
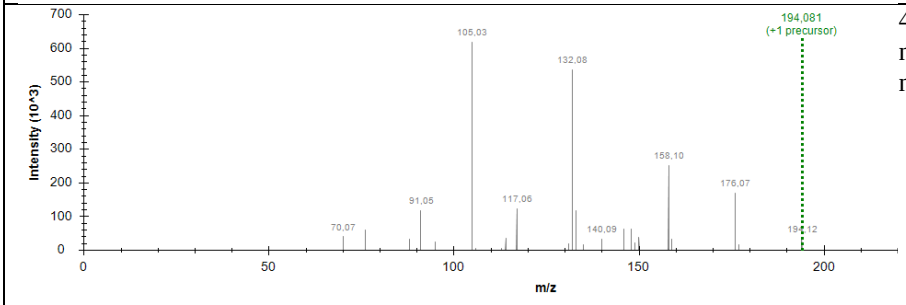
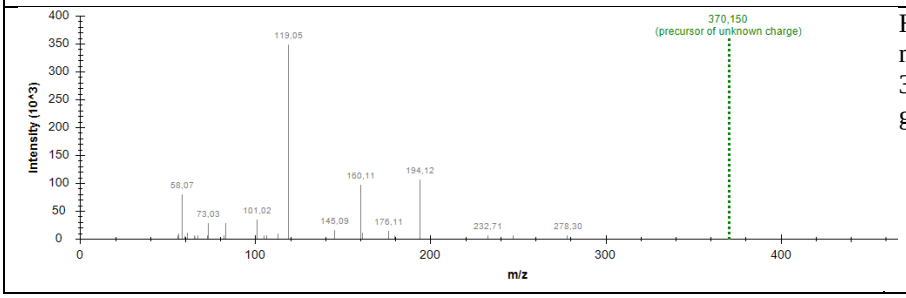
| | | | | | |
|---|---|---|---|---|---|
| **Precursor ion** | 208.0968 |  | -1.44 | - | - |
| **Fragment ion** | 144.0808 |  | -2.08 | - | - |

**Table S10** ddMS2 spectra of mephedrone metabolites detected in rat urine sample using LC Q-E.

| | |
|---|---|
|  | Mephedrone, tr: 3.4 |
|  | Normephedrone, tr: 3.3 |
|  | 4'-hydroxymethyl-mephedrone (hydroxytolyl-m.), ), tr: 1.9 |
|  | 4'-carboxy-mephedrone, tr: 1.1 |
|  | 4'-carboxy-normephedrone, tr: 1.1 |
|  | Hydroxyl-mephedrone-3O-glucuronide |

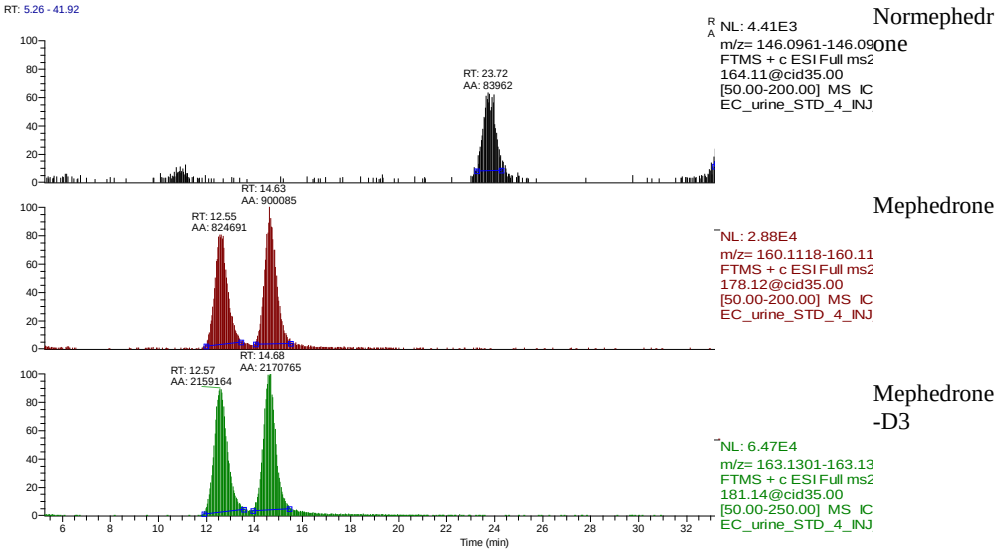



**Figure S6** MS2 chromatogram of mephedrone and normephedrone in rat urine sample using chiral LC VP.

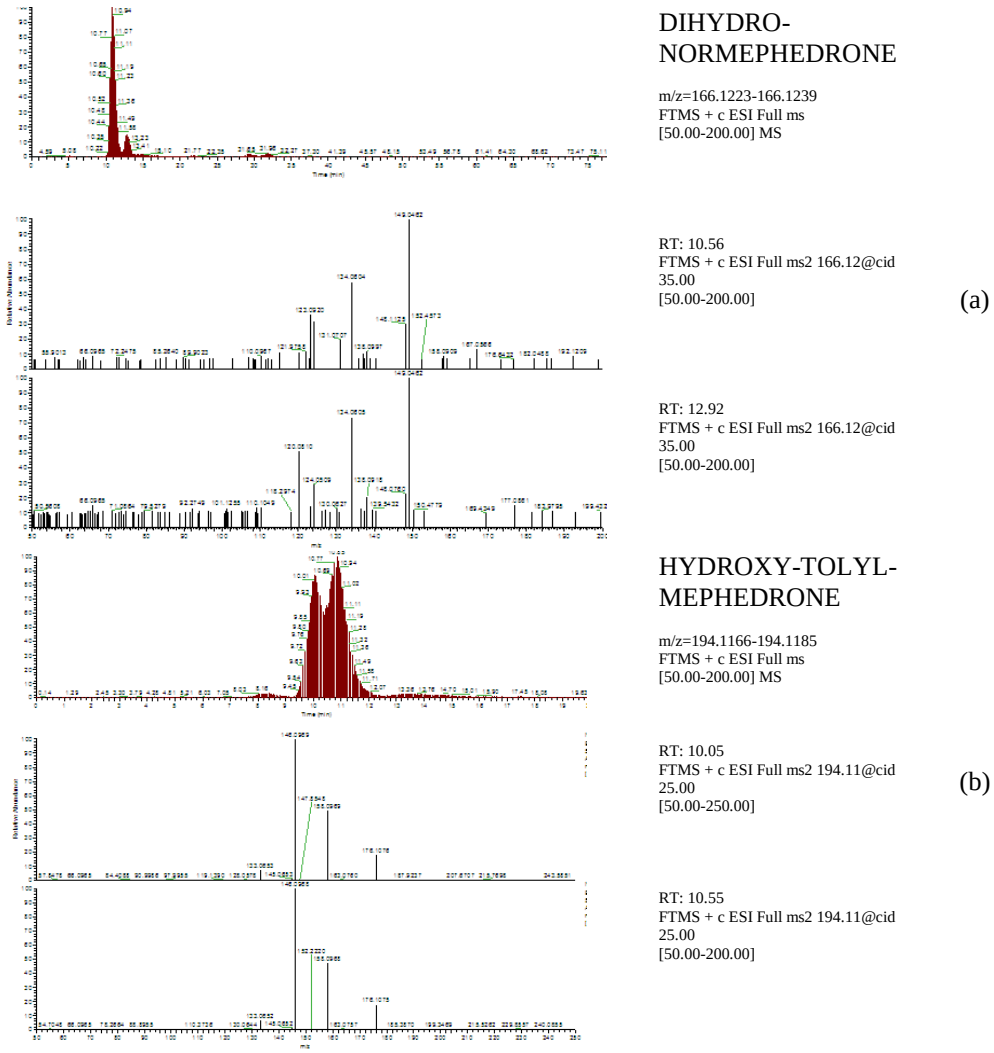



**Figure S7** Full scan chromatograms and spectra of mephedrone metabolites in rat urine sample using chiral LC VP. Dihydro-nor-mephedrone diastereoisomers are shown in (a), whilst the partial separation of hydroxytolyl-mephedrone enantiomers in (b).

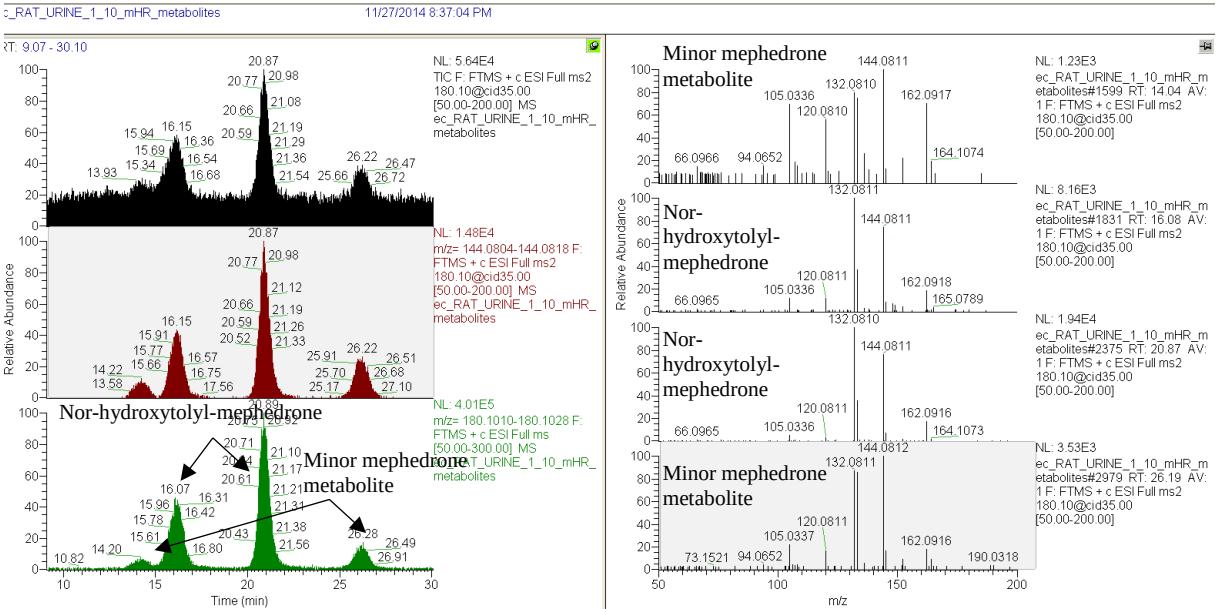
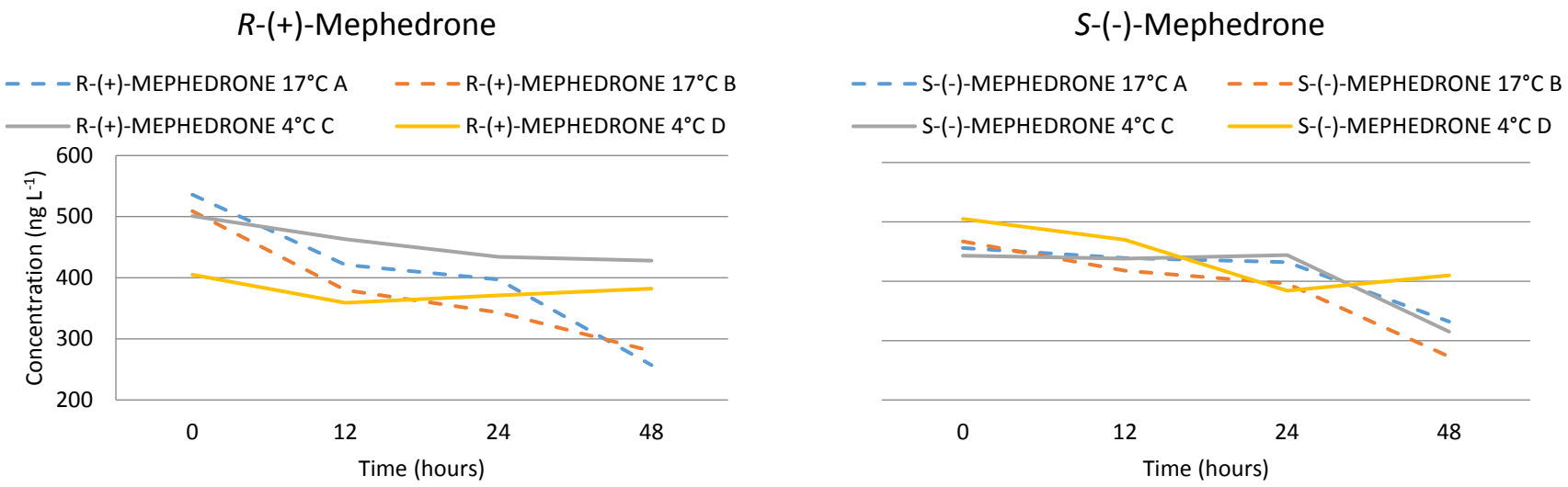
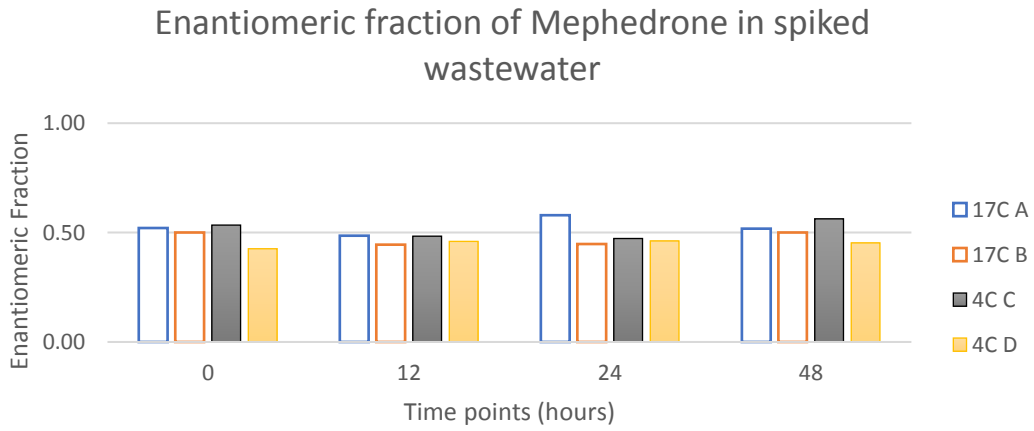



**Figure S8** Mephedrone metabolites in rat urine sample identified with chiral LC-VP: nor-hydroxytolyl-mephedrone and its two enantiomers (note: the presence of another minor mephedrone metabolite was observed).


*R*-(+)-Mephedrone
R-(+)-MEPHEDRONE 17°C A
R-(+)-MEPHEDRONE 17°C B
R-(+)-MEPHEDRONE 4°C C
R-(+)-MEPHEDRONE 4°C D
Concentration (ng L-1)
600
500
400
300
200
0
12
24
48
Time (hours)
*S*-(-)-Mephedrone
S-(-)-MEPHEDRONE 17°C A
S-(-)-MEPHEDRONE 17°C B
S-(-)-MEPHEDRONE 4°C C
S-(-)-MEPHEDRONE 4°C D
0
12
24
48
Time (hours)


Enantiomeric fraction of Mephedrone in spiked wastewater
Enantiomeric Fraction
1.00
0.50
0.00
0
12
24
48
Time points (hours)
17C A
17C B
4C C
4C D

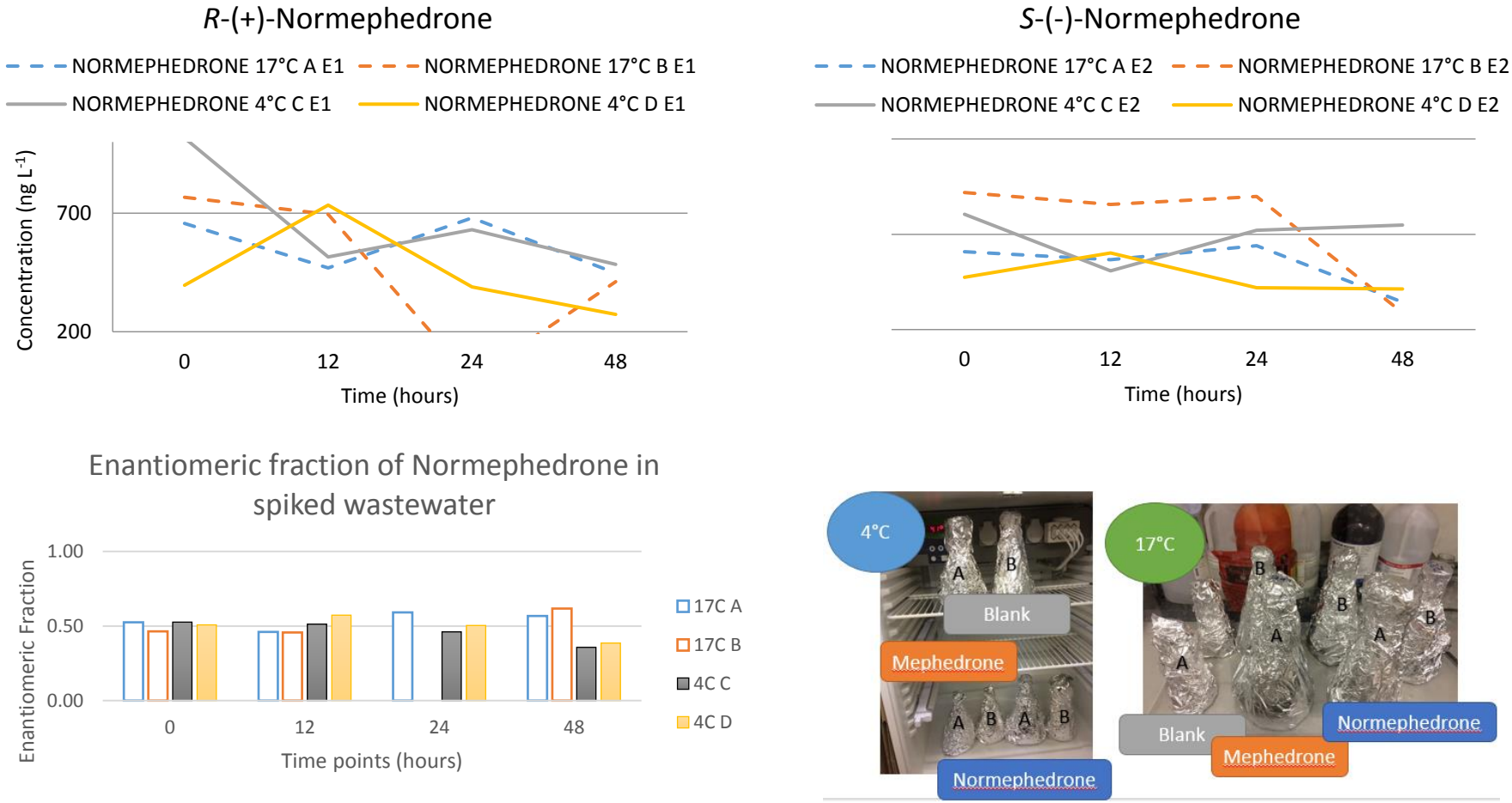



**Figure S9** Mephedrone and normephedrone concentrations and enantiomeric fraction in wastewater stability study. Picture of experimental settings.

# Appendix 3

The following supplementary data are contained in Appendix 3:

Table S1 Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, a extracted from (Moffat, Osselton et al. 2004), b predicted using ACD/labs software (http://www.chemspider.com).

Table S2 MRM transitions selected for studied analytes.

Table S3 Validation parameters - retention time, relative retention time, linearity range, correlation coefficient obtained from calibration curve and instrumental and method limits of detection and instrumental and method limits of quantification.

Table S4 Validation parameters - method precision.

Table S5 Validation parameters -instrumental precision.

Table S6 Validation parameters –ion suppression.

Table S7 SPE recovery for the studied analytes.

Table S8 Amphetamine loads.

**Table S1** Selected analytes and their properties (MW molecular weight, Exp experimental, Pred predicted, [a] extracted from (Moffat, Osselton et al. 2004), [b] predicted using ACD/labs software (http://www.chemspider.com).

| Compound | CAS | Formula | MW | $pK_a$ | | LogP | | LogD[b] | | Supplier |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Exp.[a] | Pred.[a] | Exp.[a] | Pred.[b] | pH 5.5 | pH 7.4 | |
| (±)-Amphetamine | 300-62-9 | $C_9H_{13}N$ | 135.2 | 10.1 (20°) | 10.01 | 1.85 | 1.81±0.20 | -1.28 | -0.63 | LGC(Cerilliant product) |
| (±)-Methamphetamine | 4846-07-5 | $C_{10}H_{15}N$ | 149.2 | 9.87 (25°) | 10.21 | 2.07 | 1.94±0.21 | -1.15 | -0.79 | LGC (Cerilliant product) |
| (±)-Mephedrone | 1189726-22-4 | $C_{11}H_{15}NO$ | 177.7 | - | - | - | 1.86±0.31 | -0.03 | 1.55 | Sigma-Aldrich (Cerilliant product) |
| PMA (*p*-Methoxyamphetamine) | 3706-26-1 | $C_{10}H_{15}NO$ | 165.0 | - | - | - | 1.72±0.23 | -1.36 | -0.76 | LGC |
| (±)-MDA (3,4-methylenedioxyamphetamine) | 4764-17-4 | $C_{10}H_{13}NO_2$ | 179.2 | 9.67 (25°) | 10.01 | 1.64 | 1.67±0.27 | -1.41 | -0.77 | LGC (Cerilliant product) |
| (±)-MDMA (3,4-methylenedioxymethamphetamine) | 42542-10-9 | $C_{11}H_{15}NO_2$ | 193.2 | - | 10.143 | - 1.65,1.86 | 1.81±0.27 | -1.29 | -0.90 | LGC |
| (±)-MDEA (3,4-methylenedioxyethylamphetamine) | 82801-81-8 | $C_{12}H_{17}NO_2$ | 207.3 | - | - | - | 2.66±0.27 | -0.42 | 0.30 | LGC (Cerilliant product) |
| *D,L*-HMA (*d,l*-4-Hydroxy-3-methoxyamphetamine) | 13062-61-8 | $C_{10}H_{15}NO_2$ | 181.2 | - | - | - | - | - | - | Kinesis |
| *D,L*-HMMA (*d,l*-4-Hydroxy-3-methoxymethamphetamine) | 438625-58-2 | $C_{11}H_{17}NO_2$ | 195.2 | - | - | - | 1.41 | -1.68 | -1.24 | Kinesis |
| (±)-*cis*-Tramadol | 36282-47-0 | $C_{16}H_{25}NO_2$ | 263.4 | 9.41 | 13.80, 9.23 | 2.4 | 2.51±0.28 | -0.53 | 0.49 | Sigma-Aldrich |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| (±)-Fluoxetine | 59333-67-4 | $C_{17}H_{18}F_3NO$ | 309.3 | - | 9.80 | 4.05 | 4.09±0.45 | 1.00 | 1.57 | LGC (Cerilliant product) |
| (±)-Norfluoxetine | - | $C_{16}H_{16}F_3NO$ | 295.3 | - | 9.77 | - | 4.36±0.42 | 1.39 | 2.71 | LGC (Cerilliant product) |
| (±)-Venlafaxine | 99300-78-4 | $C_{17}H_{27}NO_2$ | 277.4 | - | 14.42, 8.91 | - | 2.91±0.35 | -0.08 | 1.19 | Sigma-Aldrich |
| (±)-Desvenlafaxine | 300827-87-6 | $C_{16}H_{25}NO_2$ | 263.0 | - | 10.11, 8.87 | - | 2.26±0.34 | -0.73 | 0.52 | Sigma-Aldrich |
| (±)-Zopiclone | 43200-80-2 | $C_{17}H_{17}ClN_6O_6$ | 388.8 | - | 13.04, 6.89 | 0.80 | -0.33±1.28 | -1.55 | -0.41 | LGC |
| (±)-Ephedrine | 50-98-6 | $C_{10}H_{15}NO$ | 165.2 | 10.3 (0°) | 13.89, 9.52 | 1.13 | 1.05±0.27 | -1.99 | -0.96 | Sigma-Aldrich |
| (±)-Norephedrine | 154-41-6 | $C_9H_{13}NO$ | 151.2 | 9.44 (20°) | 13.9, 9.37 | 0.67 | 0.81±0.26 | -2.21 | -1.07 | Sigma-Aldrich |

**Table S2** MRM transitions selected for studied analytes.

| Compound | CV/CE[a] | MRM1 (quantification) | CV/CE[a] | MRM2 (confirmation) | CV/CE[a] | MRM3 (confirmation) | MRM1/MRM2 ratio ± SD | MRM1/MRM3 ratio ± SD | IS |
|---|---|---|---|---|---|---|---|---|---|
| Amphetamine | 18/16 | 136.16 > 91.1 | 18/8 | 136.16 > 119.1 | - | - | 1.2 ± 0.1 | - | Amphetamine-D5 |
| Methamphetamine | 24/19 | 150.2 > 91.1 | 24/10 | 150.2 > 119.1 | - | - | 1.8 ± 0.1 | - | Methamphetamine-D5 |
| MDA | 21/11 | 180.0 > 163.1 | 21/22 | 180.0 > 105.1 | - | - | 2.6 ± 0.4 | - | MDA-D5 |
| MDMA | 24/13 | 194.1 > 163.1 | 24/24 | 194.1 > 105.1 | - | - | 2.1 ± 0.1 | - | MDMA-D5 |
| MDEA | 28/13 | 208.1 > 163.1 | 28/27 | 208.1 > 105.1 | - | - | 2.1 ± 0.2 | - | MDEA-D5 |
| HMA | 6/14 | 182.1 > 165.0 | 6/24 | 182.1 > 105.0 | 6/18 | 182.1 > 133.0 | 1.8 ± 0.7 | 2.4 ±1.4 | Amphetamine-D5 |
| HMMA | 16/12 | 196.1 > 165.0 | 16/26 | 196.1 > 105.0 | 16/22 | 196.1 > 133.0 | 3.1 ± 0.6 | 3.8 ±0.6 | Methamphetamine-D5 |
| Mephedrone | 10/12 | 178.1 > 160.1 | 10/22 | 178.1 > 145.0 | 10/22 | 178.1 > 119.0 | 1.6 ± 0.2 | 8.5 ±2.1 | Mephedrone-D3 |
| *p*-Methoxyamphetamine (PMA) | 20/20 | 166.0 > 121.0 | 20/20 | 166.0 > 149.0 | - | - | 12.5 ± 1.5 | - | MDA-D5 |
| Tramadol | 24/17 | 264.2 > 58.1 | 24/11 | 264.2 > 246.3 | - | - | 102.1 ± 3.6 | - | Methamphetamine-D5 |
| Zopiclone | 22/18 | 389.1 > 245.0 | 22/42 | 389.1 > 217.0 | - | - | | | Zopiclone-D4 |
| Fluoxetine | 25/8 | 310.3 > 148.1 | - | - | - | - | - | - | MDMA-D5 |
| Norfluoxetine | 17/7 | 296.2 > 134.1 | - | - | - | - | - | - | MDMA-D5 |
| Venlafaxine | 27/12 | 278.2 > 58.1 | 27/12 | 278.2 > 260.1 | 27/32 | 278.2 > 121.0 | 2.7 ± 0.2 | 4.5 ±0.7 | Methamphetamine-D5 |
| Desmethylvenlafaxine | 25/24 | 264.0 > 58.1 | 25/24 | 264.0 > 107.1 | 25/20 | 264.0 > 246.3 | 12.7 ± 1.8 | 66.4 ±5.9 | Methamphetamine-D5 |
| Ephedrine | 23/12 | 166.1 > 148.1 | 23/21 | 166.1 > 133.0 | - | - | 7.4 ± 0.8 | - | 1S, 2R-(+)-ephedrine-D3 |
| Pseudoephedrine | 23/12 | 166.1 > 148.1 | 23/21 | 166.1 > 133.0 | - | - | 6.9 ± 0.6 | - | 1S, 2R-(+)-ephedrine-D3 |
| Norephedrine | 23/10 | 152.1 > 134.1 | 23/16 | 152.1 > 117.1 | - | - | 3.1 ± 0.4 | - | 1S, 2R-(+)-ephedrine-D3 |

| ISs | CV/CE[a] | MRM1 (quantification) |
|---|---|---|
| Amphetamine-$D_5$ | 22/16 | 141.0 > 92.9 |
| Methamphetamine-$D_5$ | 28/12 | 155.1 > 121.0 |
| Mephedrone-$D_3$ | 30/22 | 181.1 > 163.1 |
| MDA-$D_5$ | 21/11 | 185.1 > 168.1 |
| MDMA-$D_5$ | 26/13 | 199.1 > 165.1 |

| | | |
|---|---|---|
| MDEA-$D_5$ | 28/13 | 213.1 > 163.0 |
| Zopiclone-$D_4$ | 24/16 | 393.1 > 245.0 |
| *1S,2R*-(+)-Ephedrine-$D_3$ | 23/18 | 169.2 > 151.0 |

[a]CV, cone voltage (V); CE, collision energy (eV)

**Table S3** Validation parameters - retention time, relative retention time, linearity range, correlation coefficient obtained from calibration curve and instrumental and method limits of detection and instrumental and method limits of quantification.

| Compound | $R_t$ (min) | Rel. $R_t$ | | Sample diluent | | | WWTP influent | |
|---|---|---|---|---|---|---|---|---|
| | | | Linearity range (µg/L) | $R^2$ | $IDL_{S/N}$ (µg/L) | $IQL_{S/N}$ (µg/L) | MDL (µg/L) | MQL (µg/L) |
| *R*-(-)-Amphetamine | 15.5 ±0.3 | 0.1 | 0.125-500 | 0.9987 | 0.12 | 0.50 | 0.0008 | 0.0029 |
| *S*-(+)-Amphetamine | 22.6 ±0.4 | 0.2 | 0.125-500 | 0.9988 | 0.12 | 0.50 | 0.0008 | 0.0029 |
| *R*-(-)-Methamphetamine | 14.5 ±0.4 | 0.3 | 0.050-500 | 0.9989 | 0.05 | 0.12 | 0.0003 | 0.0006 |
| *S*-(+)-Methamphetamine | 16.5 ±0.4 | 0.3 | 0.050-500 | 0.9994 | 0.05 | 0.12 | 0.0003 | 0.0007 |
| E1-Mephedrone | 16.5 ±0.4 | 0.3 | 0.250-500 | 0.9990 | 0.25 | 0.50 | 0.0013 | 0.0026 |
| E2-Mephedrone | 21.0 ±0.5 | 0.2 | 0.250-500 | 0.9993 | 0.25 | 0.50 | 0.0007 | 0.0026 |
| *R*-(-)-MDA | 28.1 ±0.5 | 0.2 | 0.500-500 | 0.9991 | 0.50 | 2.50 | 0.0028 | 0.0140 |
| *S*-(+)-MDA | 47.4 ±0.8 | 0.4 | 0.500-500 | 0.9980 | 0.50 | 2.50 | 0.0025 | 0.0124 |
| *R*-(-)-MDMA | 21.9 ±0.5 | 0.2 | 0.050-500 | 0.9992 | 0.05 | 0.25 | 0.0003 | 0.0014 |
| *S*-(+)-MDMA | 32.9 ±0.5 | 0.1 | 0.050-500 | 0.9994 | 0.05 | 0.25 | 0.0003 | 0.0013 |
| E1-MDEA | 19.0 ±0.5 | 1.8 | 0.125-500 | 0.9994 | 0.12 | 0.25 | 0.0006 | 0.0013 |
| E2-MDEA | 21.0 ±0.5 | 0.2 | 0.125-500 | 0.9995 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| E1-HMA | 17.7 ±0.4 | 0.4 | 2.500-500 | 0.9900 | 2.50 | 5.00 | 0.0118 | 0.0236 |
| E2-HMA | 34.3 ±0.5 | 0.8 | 2.500-500 | 0.9903 | 2.50 | 5.00 | 0.0113 | 0.0225 |
| E1-HMMA | 15.9 ±0.4 | 2.5 | 0.250-500 | 0.9982 | 0.25 | 0.50 | 0.0014 | 0.0028 |
| E2-HMMA | 18.6 ±0.5 | 2.5 | 0.250-500 | 0.9974 | 0.25 | 0.50 | 0.0011 | 0.0022 |
| E1-Venlafaxine | 12.5 ±0.5 | 0.6 | 0.125-500 | 0.9980 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| E2-Venlafaxine | 15.6 ±0.5 | 2.9 | 0.125-500 | 0.9971 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| E1-Norephedrine | 13.6 ±0.3 | 0.4 | 0.125-500 | 0.9981 | 0.12 | 0.25 | 0.0006 | 0.0011 |
| E2-Norephedrine | 15.1 ±0.4 | 2.2 | 0.125-500 | 0.9983 | 0.12 | 0.25 | 0.0006 | 0.0012 |
| E1-PMA | 21.3 ±0.5 | 0.5 | 0.125-500 | 0.9964 | 0.12 | 0.25 | 0.0007 | 0.0013 |
| E2-PMA | 36. 8 ±0.4 | 1.4 | 0.125-500 | 0.9994 | 0.12 | 0.25 | 0.0006 | 0.0011 |
| D1-Tramadol | 12.6 ±0.4 | 0.6 | 0.500-500 | 0.9985 | 0.50 | 1.00 | 0.0024 | 0.0047 |
| D2-Tramadol | 13.7 ±0.5 | 0.7 | 0.500-500 | 0.9989 | 0.50 | 1.00 | 0.0029 | 0.0059 |
| (+)-Ephedrine | 12.3 ±0.3 | 0.6 | 1.000-500 | 0.9974 | 1.00 | 5.00 | 0.0059 | 0.0295 |
| (-)-Ephedrine and (-)-Ψephedrine | 13.4 ±0. | 0.5 | 0.500-1000 | 0.9975 | 0.50 | 1.00 | 0.0024 | 0.0048 |
| (+)-Ψephedrine | 32.94 ±0.8 | 1.9 | 1.000-500 | 0.9903 | 1.00 | 5.00 | 0.0056 | 0.0280 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Desmethylvenlafaxine-E1 | 15.8 ±0.4 | 0.7 | 5.000-500 | 0.9941 | 5.000 | 10.000 | 0.0249 | 0.0497 |
| Desmethylvenlafaxine-E2 | 17.2 ±0.4 | 0.6 | 5.000-500 | 0.9973 | 5.000 | 10.000 | 0.0275 | 0.0550 |
| E1-Zopiclone | 32.7 ±0.3 | 4.6 | 10.000-500 | 0.9903 | 10.000 | 50.000 | 0.0285 | 0.3125 |
| E2-Zopiclone | 59.8 ±0.4 | 5.2 | 10.000-500 | 0.9909 | 10.000 | 50.000 | 0.0326 | 0.3208 |
| *S*-(+)-Fluoxetine | 43.2 ±1.8 | 3.1 | 10.000-500 | 0.9915 | 10.000 | 50.000 | 0.0533 | 0.2664 |
| *R*-(-)-Fluoxetine | 57.2 ±2.1 | 3.3 | 10.000-500 | 0.9907 | 10.000 | 50.000 | 0.0517 | 0.2588 |
| E1-Norfluoxetine | 81.3 ±6.0 | 14.4 | 10.000-500 | 0.9916 | 10.000 | 50.000 | 0.0589 | 0.2945 |
| E2-Norfluoxetine | 87.8 ±3.5 | 12.9 | 10.000-500 | 0.9921 | 10.000 | 50.000 | 0.0612 | 0.3061 |

**Table S4** Validation parameters - method precision.

| **Analyte** | **Intra-day RSD% (n=4)** | | | | | | | | | **Inter-day RSD% (n=3)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 ng/L** D 1* | 25 ng/L D 2 | 25 ng/L D 3 | 250 ng/L D 1 | 250 ng/L D 2 | 250 ng/L D 3 | 2500 ng/L D 1 | 2500 ng/L D 2 | 2500 ng/L D 3 | 25 ng/L | 250 ng/L | 2500 ng/L |
| *R*-(-)-Amphetamine | 3.3 | 2.5 | 4.6 | 5.2 | 14.7 | 10.8 | 6.2 | 3.9 | 6.2 | 3.5 | 10.2 | 5.4 |
| *S*-(+)-Amphetamine | 3.1 | 4.3 | 12.6 | 1.4 | 6.5 | 4.7 | 3.8 | 7.0 | 7.3 | 6.7 | 4.2 | 6.0 |
| *R*-(-)-Methamphetamine | 8.9 | 6.7 | 9.3 | 3.4 | 7.0 | 8.3 | 4.8 | 5.2 | 5.4 | 8.3 | 6.2 | 5.1 |
| *S*-(+)-Methamphetamine | 6.8 | 3.6 | 15.4 | 1.2 | 5.5 | 4.0 | 2.7 | 2.9 | 4.2 | 8.6 | 3.6 | 3.3 |
| E1-Mephedrone | 9.8 | 13.7 | 14.1 | 3.6 | 6.8 | 14.6 | 3.7 | 10.0 | 5.6 | 12.5 | 8.3 | 6.4 |
| E2-Mephedrone | 10.7 | 12.0 | 4.6 | 5.2 | 12.9 | 8.4 | 9.2 | 3.7 | 2.8 | 9.1 | 8.8 | 5.2 |
| *R*-(-)-MDA | 1.7 | 6.6 | 9.7 | 3.0 | 3.4 | 5.7 | 0.1 | 7.7 | 1.1 | 6.0 | 4.0 | 3.0 |
| *S*-(+)-MDA | 4.4 | 3.8 | 7.8 | 2.6 | 6.7 | 5.3 | 7.2 | 3.7 | 4.5 | 5.3 | 4.9 | 5.1 |
| *R*-(-)-MDMA | 7.0 | 1.8 | 4.0 | 5.8 | 4.6 | 3.9 | 3.4 | 1.5 | 6.5 | 4.3 | 4.8 | 3.8 |
| *S*-(+)-MDMA | 1.0 | 1.9 | 6.9 | 0.6 | 3.1 | 2.9 | 1.2 | 2.8 | 0.7 | 3.3 | 2.2 | 1.6 |
| E1-MDEA | 6.9 | 6.2 | 3.0 | 5.1 | 8.5 | 7.8 | 4.7 | 2.2 | 4.3 | 5.4 | 7.1 | 3.7 |
| E2-MDEA | 6.0 | 6.3 | 2.8 | 1.4 | 9.2 | 4.9 | 8.3 | 1.4 | 1.7 | 5.0 | 5.2 | 3.8 |
| E1-HMA | 4.4 | 5.1 | 1.6 | 7.6 | 1.1 | 4.4 | 6.4 | 6.0 | 5.9 | 3.7 | 4.4 | 6.1 |
| E2-HMA | 5.2 | 4.8 | 12.6 | 3.8 | 2.0 | 5.0 | 7.0 | 6.5 | 6.0 | 7.5 | 3.6 | 6.5 |
| E1-HMMA | 7.4 | 7.6 | 7.5 | 2.8 | 3.8 | 6.0 | 4.1 | 2.7 | 0.3 | 7.5 | 4.2 | 2.4 |
| E2-HMMA | 4.7 | 6.4 | 3.6 | 2.1 | 2.1 | 6.2 | 2.9 | 3.1 | 3.6 | 4.9 | 3.5 | 3.2 |
| E1-Venlafaxine | 9.1 | 1.5 | 5.7 | 5.5 | 5.2 | 7.5 | 5.3 | 7.1 | 5.6 | 5.4 | 6.1 | 6.0 |
| E2-Venlafaxine | 0.0 | 4.8 | 3.1 | 4.9 | 1.4 | 7.6 | 1.5 | 4.0 | 5.2 | 2.6 | 4.6 | 3.6 |
| E1-Norephedrine | 7.3 | 3.8 | 1.3 | 2.8 | 3.0 | 7.3 | 4.4 | 3.0 | 7.4 | 4.1 | 4.3 | 5.0 |
| E2-Norephedrine | 5.7 | 4.6 | 6.3 | 3.1 | 3.9 | 6.1 | 2.2 | 2.1 | 3.5 | 5.5 | 4.3 | 2.6 |
| E1-PMA | 7.7 | 4.8 | 8.3 | 1.4 | 4.4 | 3.7 | 3.8 | 4.3 | 5.3 | 6.9 | 3.2 | 4.5 |
| E2-PMA | 6.2 | 8.8 | 11.6 | 7.8 | 4.6 | 6.6 | 1.7 | 3.9 | 2.9 | 8.9 | 6.3 | 2.8 |
| D1-Tramadol | 4.9 | 7.0 | 6.6 | 6.1 | 5.7 | 3.9 | 6.5 | 1.7 | 0.5 | 6.2 | 5.3 | 2.9 |
| D2-Tramadol | 6.2 | 9.7 | 6.1 | 4.2 | 3.2 | 4.0 | 2.5 | 3.7 | 2.5 | 7.3 | 3.8 | 2.9 |
| (+)-Ephedrine | 5.3 | 16.5 | 9.8 | 5.0 | 4.5 | 6.6 | 7.2 | 2.8 | 3.3 | 10.5 | 5.4 | 4.4 |
| (-)-Ephedrine and (-)-Ψephedrine | 8.3 | 14.8 | 5.2 | 1.8 | 0.8 | 5.4 | 5.7 | 1.0 | 3.3 | 9.4 | 2.7 | 3.3 |
| (+)-Ψephedrine | 2.8 | 2.5 | 6.2 | 5.8 | 1.3 | 9.4 | 2.9 | 2.0 | 1.7 | 3.8 | 5.5 | 2.2 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Desmethylvenlafaxine-E1 | 8.7 | 7.4 | 2.3 | 8.4 | 3.7 | 9.5 | 2.7 | 5.0 | 3.7 | 6.2 | 7.2 | 3.8 |
| Desmethylvenlafaxine-E2 | 6.4 | 8.7 | 7.4 | 3.8 | 2.8 | 5.3 | 2.3 | 4.9 | 8.2 | 7.5 | 4.0 | 5.1 |
| E1-Zopiclone | 20.0 | 17.8 | 19.5 | 14.5 | 13.2 | 19.2 | 12.6 | 7.9 | 5.6 | 19.1 | 15.6 | 8.7 |
| E2-Zopiclone | 18.7 | 18.2 | 20.4 | 17.6 | 14.8 | 6.9 | 11.4 | 5.8 | 9.8 | 19.2 | 13.1 | 9.0 |
| *S*-(+)-Fluoxetine | 19.3 | 14.2 | 1.1 | 12.9 | 18.2 | 14.0 | 3.5 | 5.4 | 2.9 | 11.5 | 15.0 | 4.0 |
| *R*-(-)-Fluoxetine | 19.2 | 2.7 | 20.7 | 6.2 | 3.8 | 0.5 | 0.3 | 2.7 | 14.5 | 14.2 | 3.5 | 5.8 |
| E1-Norfluoxetine | 17.6 | 15.1 | 9.7 | 6.6 | 3.8 | 9.6 | 2.5 | 7.9 | 13.5 | 14.1 | 6.7 | 8.0 |
| E2-Norfluoxetine | 1.9 | 6.8 | 10.7 | 20.3 | 10.5 | 3.4 | 0.7 | 10.7 | 7.3 | 6.5 | 11.4 | 6.2 |

*-D indicates day

**Table S5** Validation parameters -instrumental precision.

| **Analyte** | **Intra-day RSD% (n=4)** | | | | | | | | | **Inter-day RSD% (n=3)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 µg/L** D 1* | 5 µg/L D 2 | 5 µg/L D 3 | 50 µg/L D 1 | 50 µg/L D 2 | 50 µg/L D 3 | 500 µg/L D 1 | 500 µg/L D 2 | 500 µg/L D 3 | 5 µg/L | 50 µg/L | 500 µg/L |
| *R*-(-)-Amphetamine | 4.8 | 5.8 | 3.0 | 2.3 | 3.1 | 0.1 | 3.9 | 4.7 | 3.1 | 4.5 | 1.9 | 3.9 |
| *S*-(+)-Amphetamine | 3.7 | 5.3 | 6.5 | 4.6 | 3.3 | 4.3 | 3.2 | 4.1 | 3.4 | 5.2 | 4.1 | 3.6 |
| *R*-(-)-Methamphetamine | 6.0 | 5.8 | 6.3 | 3.9 | 5.5 | 2.3 | 3.0 | 5.1 | 2.8 | 6.0 | 3.9 | 3.7 |
| *S*-(+)-Methamphetamine | 2.4 | 2.3 | 7.7 | 2.7 | 0.7 | 2.1 | 1.1 | 4.8 | 3.4 | 4.1 | 1.8 | 3.1 |
| E1-Mephedrone | 9.3 | 6.7 | 5.5 | 1.9 | 5.7 | 5.4 | 2.9 | 5.5 | 4.4 | 7.1 | 4.3 | 4.3 |
| E2-Mephedrone | 3.5 | 6.7 | 1.1 | 3.6 | 2.5 | 2.7 | 9.3 | 4.3 | 2.2 | 3.8 | 3.0 | 5.2 |
| *R*-(-)-MDA | 6.9 | 1.3 | 2.7 | 0.4 | 5.6 | 0.1 | 1.5 | 0.3 | 1.6 | 3.6 | 2.1 | 1.1 |
| *S*-(+)-MDA | 5.7 | 3.2 | 6.4 | 8.0 | 8.9 | 3.1 | 0.3 | 1.1 | 6.1 | 5.1 | 6.7 | 2.5 |
| *R*-(-)-MDMA | 2.5 | 5.5 | 2.0 | 1.8 | 6.4 | 3.9 | 4.8 | 3.7 | 6.1 | 3.3 | 4.0 | 4.9 |
| *S*-(+)-MDMA | 3.5 | 1.1 | 4.3 | 0.5 | 1.8 | 1.3 | 2.5 | 1.5 | 2.7 | 3.0 | 1.2 | 2.3 |
| E1-MDEA | 8.6 | 5.3 | 5.9 | 2.2 | 3.8 | 1.1 | 6.1 | 4.3 | 0.1 | 6.6 | 2.4 | 3.5 |
| E2-MDEA | 3.6 | 2.3 | 10.3 | 5.3 | 1.1 | 0.4 | 5.6 | 1.9 | 0.7 | 5.4 | 2.3 | 2.7 |
| E1-HMA | 11.3 | 5.6 | 6.3 | 5.3 | 6.7 | 9.1 | 7.4 | 4.9 | 2.1 | 7.7 | 7.1 | 4.8 |
| E2-HMA | 6.1 | 1.7 | 1.1 | 3.1 | 0.4 | 2.5 | 8.9 | 6.3 | 9.0 | 3.0 | 2.0 | 8.1 |
| E1-HMMA | 5.3 | 8.3 | 4.1 | 0.8 | 6.5 | 6.6 | 8.2 | 4.2 | 1.7 | 5.9 | 4.6 | 4.7 |
| E2-HMMA | 6.6 | 5.7 | 9.4 | 2.4 | 3.3 | 7.4 | 3.8 | 4.0 | 4.6 | 7.2 | 4.4 | 4.1 |
| E1-Venlafaxine | 7.1 | 3.1 | 7.4 | 3.4 | 2.0 | 0.8 | 0.4 | 1.4 | 0.9 | 5.9 | 2.1 | 0.9 |
| E2-Venlafaxine | 6.1 | 2.4 | 0.0 | 1.9 | 2.9 | 3.6 | 0.5 | 1.4 | 0.9 | 2.8 | 2.8 | 0.9 |
| E1-Norephedrine | 5.9 | 7.1 | 3.1 | 2.7 | 6.9 | 5.7 | 2.2 | 1.3 | 1.1 | 5.4 | 5.1 | 1.5 |
| E2-Norephedrine | 5.4 | 3.1 | 4.4 | 2.4 | 4.7 | 3.6 | 3.4 | 5.2 | 2.1 | 4.3 | 3.6 | 3.6 |
| E1-PMA | 2.7 | 8.4 | 5.6 | 1.2 | 8.6 | 6.1 | 2.6 | 0.3 | 0.4 | 5.6 | 5.3 | 1.1 |
| E2-PMA | 4.6 | 5.4 | 4.5 | 4.6 | 3.9 | 2.6 | 3.1 | 1.6 | 1.1 | 4.9 | 3.7 | 1.9 |
| D1-Tramadol | 11.2 | 7.1 | 5.6 | 3.4 | 4.1 | 3.6 | 5.1 | 0.9 | 2.5 | 8.0 | 3.7 | 2.8 |
| D2-Tramadol | 2.4 | 6.8 | 10.7 | 13.8 | 12.4 | 7.1 | 2.2 | 10.0 | 1.1 | 6.7 | 11.1 | 4.4 |
| (+)-Ephedrine | 6.9 | 3.5 | 6.5 | 4.3 | 5.7 | 3.2 | 6.3 | 1.0 | 3.4 | 5.6 | 4.4 | 3.6 |
| (-)-Ephedrine and (-)-Ψephedrine | 2.6 | 2.7 | 4.1 | 3.6 | 3.1 | 2.3 | 6.4 | 0.7 | 4.4 | 3.1 | 3.0 | 3.8 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (+)-Ψephedrine | 10.6 | 6.2 | 5.6 | 5.9 | 0.5 | 2.4 | 3.4 | 9.1 | 3.2 | 7.4 | 2.9 | 5.3 |
| Desmethylvenlafaxine-E1 | 18.2 | 7.4 | 5.2 | 5.3 | 7.2 | 0.7 | 1.9 | 8.1 | 2.0 | 10.3 | 4.4 | 4.0 |
| Desmethylvenlafaxine-E2 | 5.4 | 4.5 | 4.0 | 5.7 | 12.5 | 6.4 | 2.8 | 2.0 | 3.9 | 4.7 | 8.2 | 2.9 |
| E1-Zopiclone | 18.2 | 19.7 | 15.8 | 14.2 | 16.2 | 12.2 | 11.4 | 10.7 | 8.7 | 17.9 | 14.2 | 10.3 |
| E2-Zopiclone | 17.9 | 19.3 | 16.7 | 12.6 | 18.6 | 18.0 | 14.6 | 13.8 | 8.5 | 17.9 | 16.4 | 12.3 |
| *S*-(+)-Fluoxetine | 18.3 | 6.9 | 4.9 | 6.0 | 19.0 | 9.3 | 13.6 | 14.4 | 2.4 | 10.0 | 11.4 | 10.2 |
| *R*-(-)-Fluoxetine | 16.5 | 15.9 | 10.2 | 13.0 | 1.3 | 16.9 | 1.7 | 9.3 | 2.4 | 14.2 | 10.4 | 4.5 |
| E1-Norfluoxetine | 11.5 | 4.4 | 18.4 | 5.9 | 8.2 | 0.8 | 5.1 | 3.1 | 3.4 | 11.4 | 5.0 | 3.9 |
| E2-Norfluoxetine | 10.2 | 14.3 | 16.6 | 15.5 | 9.8 | 13.8 | 17.6 | 8.0 | 0.5 | 13.7 | 13.1 | 8.7 |

*-D indicates day

**- the following concentrations were used: 10, 100 and 1000 ng/L in the case of compounds that were not enantioseparated

**Table S6** Validation parameters –ion suppression.

| Analyte | Signal suppression (%) (n=4) |
|---|---|
| *R*-(-)-Amphetamine | 37.9 ± 9.7 |
| *S*-(+)-Amphetamine | 53.4 ± 9.4 |
| *R*-(-)-Methamphetamine | -28.5 ± 12.5 |
| *S*-(+)-Methamphetamine | -6.4 ± 9.2 |
| E1-Mephedrone | -22.3 ± 11.8 |
| E2-Mephedrone | -40.2 ± 14.0 |
| *R*-(-)-MDA | -15.3 ± 1.4 |
| *S*-(+)-MDA | -12.5 ± 1.8 |
| *R*-(-)-MDMA | -43.9 ± 7.4 |
| *S*-(+)-MDMA | -57.5 ± 8.2 |
| E1-MDEA | -33.9 ± 2.0 |
| E2-MDEA | -58.1 ± 6.6 |
| E1-HMA | -50.4 ± 6.2 |
| E2-HMA | -68.7 ± 13.9 |
| E1-HMMA | -81.5 ± 33.7 |
| E2-HMMA | -76.7 ± 15.0 |
| E1-Venlafaxine | -19.3 ± 4.8 |
| E2-Venlafaxine | -12.1 ± 9.5 |
| E1-Norephedrine | 63.4 ± 2.8 |
| E2-Norephedrine | 21.5 ± 4.6 |
| E1-PMA | -21.7 ± 7.9 |
| E2-PMA | -38.8 ± 4.1 |
| D1-Tramadol | 22.1 ± 1.5 |
| D2-Tramadol | 8.8 ± 6.6 |
| (+)-Ephedrine | -78.2 ± 4.6 |
| (-)-Ephedrine and (-)-Ψephedrine | -72.3 ± 7.5 |
| (+)-Ψephedrine | -76.7 ± 16.3 |
| Desmethylvenlafaxine-E1 | -6.3 ± 2.0 |
| Desmethylvenlafaxine-E2 | -31.1 ± 16.4 |
| E1-Zopiclone | -33.2 ± 5.6 |
| E2-Zopiclone | -41.0 ± 4.5 |
| *S*-(+)-Fluoxetine | 1.5 ± 0.1 |
| *R*-(-)-Fluoxetine | 3.2 ± 2.5 |
| E1-Norfluoxetine | -4.3 ± 0.7 |
| E2-Norfluoxetine | -11.0 ± 0.8 |

**Table S7** SPE recovery for the studied analytes.

| | SPE relative recovery % (n=3) | | |
|---|---|---|---|
| **Analyte** | 25 ng/L* | 250 ng/L* | 2500 ng/L* |
| *R*-(-)-Amphetamine | 101.0 ± 6.6 | 76.0 ± 1.6 | 82.0 ± 4.7 |
| *S*-(+)-Amphetamine | 81.0 ± 10.6 | 99.0 ± 2.0 | 82.0 ± 4.2 |
| *R*-(-)-Methamphetamine | 91.0 ± 4.4 | 113.0 ± 0.7 | 82.0 ± 5.0 |
| *S*-(+)-Methamphetamine | 84.0 ± 1.9 | 86.0 ± 1.2 | 84.0 ± 7.1 |
| E1-Mephedrone | 109.0 ± 3.2 | 99.0 ± 4.8 | 80.0 ± 7.0 |
| E2-Mephedrone | 99.0 ± 8.5 | 99.0 ± 4.3 | 87.0 ± 11.5 |
| *R*-(-)-MDA | 93.0 ± 6.2 | 94.0 ± 4.2 | 81.0 ± 1.0 |
| *S*-(+)-MDA | 110.0 ± 8.5 | 99.0 ± 1.5 | 91.0 ± 1.5 |
| *R*-(-)-MDMA | 91.0 ± 3.7 | 81.0 ± 7.8 | 89.0 ± 4.3 |
| *S*-(+)-MDMA | 93.0 ± 1.7 | 100.0 ± 0.7 | 84.0 ± 1.9 |
| E1-MDEA | 102.0 ± 2.0 | 95.0 ± 8.6 | 91.0 ± 5.9 |
| E2-MDEA | 99.0 ± 1.8 | 92.0 ± 1.9 | 93.0 ± 13.4 |
| E1-HMA | 97.0 ± 8.7 | 114.0 ± 0.3 | 106.0 ± 16.4 |
| E2-HMA | 106.0 ± 4.6 | 107.0 ± 2.9 | 120.0 ± 11.5 |
| E1-HMMA | 84.0 ± 8.8 | 85.0 ± 9.4 | 100.0 ± 3.3 |
| E2-HMMA | 108.0 ± 7.5 | 105.0 ± 2.4 | 118.0 ± 1.7 |
| E1-Venlafaxine | 83.0 ± 0.6 | 105.0 ± 6.3 | 91.0 ± 0.4 |
| E2-Venlafaxine | 91.0 ± 5.8 | 104.0 ± 5.4 | 90.0 ± 0.7 |
| E1-Norephedrine | 112.0 ± 2.8 | 117.0 ± 1.1 | 108.0 ± 1.5 |
| E2-Norephedrine | 115.0 ± 5.9 | 95.0 ± 2.1 | 83.0 ± 1.4 |
| E1-PMA | 110.0 ± 8.5 | 94.0 ± 2.4 | 80.0 ± 0.7 |
| E2-PMA | 113.0 ± 3.5 | 118.0 ± 5.9 | 91.0 ± 0.4 |
| D1-Tramadol | 109.0 ± 6.0 | 111.0 ± 7.2 | 96.0 ± 10.0 |
| D2-Tramadol | 90.0 ± 7.8 | 81.0 ± 2.7 | 80.0 ± 1.1 |
| (+)-Ephedrine | 81.0 ± 9.0 | 82.0 ± 2.6 | 91.0 ± 2.1 |
| (-)-Ephedrine and (-)-Ψephedrine | 112.0 ± 0.6 | 87.0 ± 2.5 | 113.0 ± 9.6 |
| (+)-Ψephedrine | 104.0 ± 10.6 | 83.0 ± 0.3 | 81.0 ± 1.0 |
| Desmethylvenlafaxine-E1 | 91.0 ± 9.8 | 113.0 ± 14.2 | 98.0 ± 6.5 |
| Desmethylvenlafaxine-E2 | 82.0 ± 1.1 | 92.0 ± 4.1 | 99.0 ± 10.7 |
| E1-Zopiclone | 80.0 ± 2.0 | 82.0 ± 0.7 | 81.0 ± 3.7 |
| E2-Zopiclone | 80.0 ± 1.2 | 80.0 ± 6.7 | 83.0 ± 4.6 |
| *S*-(+)-Fluoxetine | 100.0 ± 5.5 | 81.0 ± 3.8 | 100.0 ± 0.7 |
| *R*-(-)-Fluoxetine | 97.0 ± 16.6 | 91.0 ± 5.5 | 101.0 ± 7.1 |
| E1-Norfluoxetine | 87.0 ± 1.7 | 80.0 ± 4.6 | 87.0 ± 5.3 |
| E2-Norfluoxetine | 80.0 ± 0.4 | 81.0 ± 1.7 | 84.0 ± 2.6 |

**Table S8** Amphetamine loads.

| R-(+)-Amphetamine | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
| Mon | 46.2 | 152.5 | 70.6 | 233.0 | 1.1 | 3.6 | 8.8 | 29.1 | - | - | 39.6 | 130.7 | 14.3 | 47.3 | 40.5 | 133.6 |
| Tues | 48.1 | 158.7 | 56.4 | 186.0 | - | - | 30.6 | 100.9 | - | - | 29.4 | 97.0 | 12.7 | 41.8 | 41.4 | 136.7 |
| Wed | 45.3 | 149.6 | 76.3 | 251.7 | - | - | 27.4 | 90.4 | - | - | 27.6 | 91.2 | 13.3 | 44.0 | 39.3 | 129.8 |
| Thur | 42.4 | 140.0 | 75.0 | 247.6 | 0.7 | 2.5 | 16.5 | 54.3 | - | - | 29.3 | 96.6 | 13.2 | 43.7 | 46.8 | 154.3 |
| Fri | 46.6 | 153.8 | 76.3 | 251.9 | - | - | 22.1 | 73.1 | - | - | 33.8 | 111.4 | 14.7 | 48.6 | 45.8 | 151.1 |
| Sat | 50.4 | 166.3 | 76.4 | 252.3 | - | - | 26.6 | 87.8 | - | - | 36.5 | 120.5 | 15.8 | 52.0 | 46.4 | 153.3 |
| Sun | 44.6 | 147.0 | 46.8 | 154.4 | 1.0 | 3.2 | 7.9 | 25.9 | - | - | 44.1 | 145.4 | 19.9 | 65.5 | 46.7 | 154.0 |
| AV | 46.2 | 152.6 | 68.3 | 225.3 | 0.9 | 3.1 | 20.0 | 65.9 | - | - | 34.3 | 113.3 | 14.8 | 49.0 | 43.8 | 144.7 |
| SD | 2.5 | 8.4 | 11.9 | 39.3 | 0.2 | 0.6 | 9.1 | 30.1 | - | - | 6.1 | 20.1 | 2.4 | 8.1 | 3.3 | 10.8 |
| CV | 0.06 | 0.06 | 0.17 | 0.17 | 0.18 | 0.18 | 0.46 | 0.46 | - | - | 0.18 | 0.18 | 0.16 | 0.16 | 0.07 | 0.07 |
| **S-(+)-Amphetamine** | | | | | | | | | | | | | | | | |
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |

| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mon | 34.1 | 112.5 | 60.7 | 200.4 | 3.5 | 11.7 | 7.4 | 24.4 | - | - | 27.6 | 91.0 | 16.5 | 54.3 | 32.5 | 107.2 |
| Tue | 39.3 | 129.8 | 32.2 | 106.3 | - | - | 24.5 | 81.0 | - | - | 20.5 | 67.6 | 12.7 | 42.0 | 33.1 | 109.4 |
| Wed | 38.3 | 126.2 | 60.7 | 200.3 | - | - | 22.0 | 72.8 | - | - | 19.5 | 64.3 | 12.8 | 42.4 | 28.4 | 93.7 |
| Thur | 32.7 | 108.1 | 54.0 | 178.1 | 1.1 | 3.7 | 13.7 | 45.2 | - | - | 20.1 | 66.5 | 12.6 | 41.7 | 34.7 | 114.5 |
| Fri | 39.2 | 129.3 | 60.3 | 199.1 | - | - | 18.3 | 60.5 | - | - | 22.3 | 73.6 | 9.1 | 29.9 | 31.8 | 104.9 |
| Sat | 39.2 | 129.4 | 65.7 | 216.9 | - | - | 22.4 | 73.9 | - | - | 25.8 | 85.0 | 17.5 | 57.6 | 33.7 | 111.2 |
| Sun | 32.0 | 105.7 | 44.6 | 147.3 | 1.2 | 3.8 | 6.7 | 22.2 | - | - | 31.9 | 105.3 | 19.7 | 64.9 | 34.5 | 113.9 |
| AV | 36.4 | 120.1 | 54.0 | 178.3 | 1.9 | 6.4 | 16.5 | 54.3 | - | - | 24.0 | 79.0 | 14.4 | 47.5 | 32.7 | 107.8 |
| SD | 3.3 | 10.9 | 11.8 | 38.8 | 1.4 | 4.6 | 7.3 | 24.1 | - | - | 4.6 | 15.3 | 3.6 | 11.9 | 2.2 | 7.1 |
| CV | 0.09 | 0.09 | 0.22 | 0.22 | 0.71 | 0.71 | 0.44 | 0.44 | - | - | 0.19 | 0.19 | 0.25 | 0.25 | 0.07 | 0.07 |
| **(±)-Amphetamine** | | | | | | | | | | | | | | | | |
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
| Mon | 80.3 | 265.0 | 131.3 | 433.3 | 4.6 | 15.2 | 16.2 | 53.6 | - | - | 67.2 | 221.8 | 30.8 | 101.7 | 73.0 | 240.9 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tue | 87.4 | 288.5 | 88.6 | 292.3 | - | - | 55.1 | 181.9 | - | - | 49.9 | 164.5 | 25.4 | 83.8 | 74.6 | 246.0 |
| Wed | 83.6 | 275.8 | 137.0 | 452.0 | - | - | 49.4 | 163.1 | - | - | 47.1 | 155.5 | 26.2 | 86.4 | 67.7 | 223.4 |
| Thur | 75.2 | 248.1 | 129.0 | 425.8 | 1.9 | 6.2 | 30.2 | 99.5 | - | - | 49.4 | 163.1 | 25.9 | 85.4 | 81.4 | 268.8 |
| Fri | 85.8 | 283.1 | 136.7 | 451.0 | - | - | 40.5 | 133.6 | - | - | 56.1 | 185.1 | 23.8 | 78.5 | 77.6 | 256.0 |
| Sat | 89.6 | 295.7 | 142.2 | 469.2 | - | - | 49.0 | 161.8 | - | - | 62.3 | 205.5 | 33.2 | 109.6 | 80.1 | 264.4 |
| Sun | 76.6 | 252.7 | 91.4 | 301.7 | 2.1 | 7.0 | 14.6 | 48.1 | - | - | 76.0 | 250.7 | 39.5 | 130.5 | 81.2 | 267.9 |
| AV | 82.6 | 272.7 | 122.3 | 403.6 | 2.9 | 9.5 | 36.4 | 120.2 | - | - | 58.3 | 192.3 | 29.3 | 96.5 | 76.5 | 252.5 |
| SD | 5.5 | 18.1 | 22.5 | 74.2 | 1.5 | 5.0 | 16.4 | 54.2 | - | - | 10.7 | 35.3 | 5.6 | 18.6 | 5.1 | 16.7 |
| CV | 0.07 | 0.07 | 0.18 | 0.18 | 0.53 | 0.53 | 0.45 | 0.45 | - | - | 0.18 | 0.18 | 0.19 | 0.19 | 0.07 | 0.07 |

**Table S9** Methamphetamine loads.

| R-(+)-Methamphetamine | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
| Mon | 0.4 | 1.0 | 172.3 | 396.2 | 0.0 | 0.0 | - | - | - | - | - | - | - | - | - | - |
| Tue | 0.7 | 1.6 | 113.0 | 259.8 | 0.7 | 1.6 | - | - | - | - | - | - | - | - | - | - |
| Wed | 0.8 | 1.8 | 61.4 | 141.3 | 0.9 | 2.0 | - | - | - | - | - | - | - | - | - | - |
| Thur | 0.7 | 1.5 | 91.9 | 211.3 | 1.4 | 3.2 | - | - | - | - | - | - | - | - | - | - |
| Fri | 0.0 | 0.0 | 60.4 | 139.0 | 0.0 | 0.0 | - | - | - | - | - | - | - | - | - | - |
| Sat | 2.1 | 4.9 | 96.1 | 220.9 | 0.0 | 0.0 | - | - | - | - | - | - | - | - | - | - |
| Sun | 0.8 | 1.8 | 56.1 | 129.1 | 0.0 | 0.0 | - | - | - | - | - | - | - | - | - | - |
| AV | 0.8 | 1.8 | 93.0 | 214.0 | 0.4 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 0.6 | 1.5 | 41.0 | 94.4 | 0.6 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 0.83 | 0.83 | 0.44 | 0.44 | 1.34 | 1.34 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| **S-(+)-Methamphetamine** | | | | | | | | | | | | | | | | |
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
| | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION |

| | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mon | 0.4 | 1.0 | 146.3 | 336.4 | 8.6 | 19.9 | - | - | - | - | 4.0 | 9.2 | 21.6 | 49.6 | 7.7 | 17.8 |
| Tue | 0.3 | 0.8 | 94.7 | 217.7 | 11.9 | 27.3 | - | - | - | - | 3.1 | 7.2 | 18.1 | 41.6 | 6.4 | 14.7 |
| Wed | 0.4 | 1.0 | 53.1 | 122.2 | 8.7 | 20.0 | - | - | - | - | 3.9 | 8.9 | 18.0 | 41.5 | 6.7 | 15.3 |
| Thur | 0.4 | 1.0 | 86.8 | 199.6 | 8.3 | 19.0 | - | - | - | - | 3.3 | 7.7 | 15.8 | 36.4 | 5.1 | 11.7 |
| Fri | 0.0 | 0.0 | 51.1 | 117.6 | 9.5 | 21.8 | - | - | - | - | 3.9 | 9.1 | 18.3 | 42.2 | 5.5 | 12.7 |
| Sat | 0.6 | 1.4 | 79.1 | 181.8 | 10.4 | 23.9 | - | - | - | - | 3.8 | 8.7 | 23.0 | 52.8 | 8.1 | 18.7 |
| Sun | 0.4 | 1.0 | 44.3 | 102.0 | 11.6 | 26.6 | - | - | - | - | 3.6 | 8.3 | 26.5 | 60.9 | 6.6 | 15.3 |
| AV | 0.4 | 0.9 | 79.3 | 182.5 | 9.8 | 22.6 | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 8.4 | 20.2 | 46.4 | 6.6 | 15.2 |
| SD | 0.2 | 0.4 | 35.3 | 81.2 | 1.5 | 3.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.7 | 3.7 | 8.4 | 1.1 | 2.5 |
| CV | 0.49 | 0.49 | 0.44 | 0.44 | 0.15 | 0.15 | 0.0 | 0.0 | 0.0 | 0.0 | 0.09 | 0.09 | 0.18 | 0.18 | 0.17 | 0.17 |

**(±)-Methamphetamine**

| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
| Mon | 0.9 | 2.0 | 318.5 | 732.6 | 8.6 | 19.9 | - | - | - | - | 4.0 | 9.2 | 21.6 | 49.6 | 7.7 | 17.8 |
| Tue | 1.0 | 2.4 | 207.6 | 477.5 | 12.6 | 28.9 | - | - | - | - | 3.1 | 7.2 | 18.1 | 41.6 | 6.4 | 14.7 |
| Wed | 1.2 | 2.8 | 114.6 | 263.5 | 9.5 | 22.0 | - | - | - | - | 3.9 | 8.9 | 18.0 | 41.5 | 6.7 | 15.3 |
| Thur | 1.1 | 2.6 | 178.7 | 410.9 | 9.6 | 22.2 | - | - | - | - | 3.3 | 7.7 | 15.8 | 36.4 | 5.1 | 11.7 |
| Fri | 0.0 | 0.0 | 111.6 | 256.6 | 9.5 | 21.8 | - | - | - | - | 3.9 | 9.1 | 18.3 | 42.2 | 5.5 | 12.7 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sat | 2.7 | 6.3 | 175.1 | 402.8 | 10.4 | 23.9 | - | - | - | - | 3.8 | 8.7 | 23.0 | 52.8 | 8.1 | 18.7 |
| Sun | 1.2 | 2.8 | 100.5 | 231.1 | 11.6 | 26.6 | - | - | - | - | 3.6 | 8.3 | 26.5 | 60.9 | 6.6 | 15.3 |
| AV | 1.2 | 2.7 | 172.4 | 396.4 | 10.3 | 23.6 | 0.0 | 0.0 | 0.0 | 0.0 | 3.7 | 8.4 | 20.2 | 46.4 | 6.6 | 15.2 |
| SD | 0.8 | 1.9 | 76.2 | 175.3 | 1.4 | 3.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.7 | 3.7 | 8.4 | 1.1 | 2.5 |
| CV | 0.69 | 0.69 | 0.44 | 0.44 | 0.13 | 0.13 | 0.0 | 0.0 | 0.0 | 0.0 | 0.09 | 0.09 | 0.18 | 0.18 | 0.17 | 0.17 |

**Table S10** MDMA loads.

| R-(-)-MDMA | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
| Mon | 36.3 | 54.5 | 40.3 | 60.5 | 4.9 | 7.3 | 29.0 | 43.5 | 2.3 | 3.4 | 19.1 | 28.6 | 41.5 | 62.2 | 22.4 | 33.6 |
| Tue | 17.6 | 26.5 | 19.0 | 28.5 | 0.0 | 0.0 | 56.5 | 84.8 | 1.8 | 2.7 | 7.6 | 11.3 | 14.9 | 22.4 | 11.9 | 17.9 |
| Wed | 14.1 | 21.2 | 14.1 | 21.2 | 1.8 | 2.7 | 34.2 | 51.3 | 0.0 | 0.0 | 6.3 | 9.5 | 7.7 | 11.5 | 7.2 | 10.8 |
| Thur | 9.9 | 14.9 | 22.7 | 34.0 | 0.7 | 1.1 | 11.2 | 16.8 | 0.0 | 0.0 | 5.6 | 8.5 | 5.5 | 8.2 | 7.6 | 11.5 |
| Fri | 20.2 | 30.4 | 11.3 | 16.9 | 2.3 | 3.5 | 37.3 | 56.0 | 2.0 | 3.0 | 6.6 | 10.0 | 8.7 | 13.0 | 9.0 | 13.5 |
| Sat | 66.0 | 98.9 | 26.8 | 40.2 | 7.8 | 11.7 | 54.3 | 81.4 | 1.5 | 2.3 | 15.6 | 23.4 | 27.8 | 41.7 | 31.1 | 46.7 |
| Sun | 69.3 | 103.9 | 30.7 | 46.0 | 13.1 | 19.7 | 43.5 | 65.2 | 2.4 | 3.7 | 35.9 | 53.8 | 91.9 | 137.8 | 45.2 | 67.8 |
| AV | 33.4 | 50.0 | 23.6 | 35.3 | 4.4 | 6.6 | 38.0 | 57.0 | 2.0 | 2.2 | 13.8 | 20.7 | 28.3 | 42.4 | 19.2 | 28.8 |
| SD | 24.8 | 37.3 | 10.0 | 15.0 | 4.7 | 7.0 | 15.6 | 23.3 | 0.4 | 1.5 | 11.0 | 16.6 | 30.9 | 46.3 | 14.5 | 21.8 |
| CV | 0.7 | 0.7 | 0.43 | 0.43 | 1.07 | 1.07 | 0.41 | 0.41 | 0.18 | 0.71 | 0.80 | 0.80 | 1.1 | 1.1 | 0.8 | 0.8 |
| **S-(+)-MDMA** | | | | | | | | | | | | | | | | |
| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
| | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/100 | ESTIMATED CONSUMPTION |

| | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | | 0 people/day) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Mon | 14.4 | 21.6 | 29.0 | 43.5 | 2.4 | 3.6 | 15.6 | 23.4 | 1.0 | 1.6 | 8.5 | 12.8 | 16.0 | 24.0 | 10.4 | 15.5 |
| Tue | 7.7 | 11.6 | 10.6 | 15.9 | 2.8 | 4.3 | 30.3 | 45.4 | 1.0 | 1.6 | 3.3 | 4.9 | 5.1 | 7.7 | 6.3 | 9.4 |
| Wed | 8.8 | 13.1 | 10.6 | 15.9 | 1.2 | 1.8 | 17.1 | 25.6 | 0.0 | 0.0 | 3.3 | 5.0 | 2.7 | 4.0 | 4.4 | 6.7 |
| Thur | 6.3 | 9.5 | 7.6 | 11.3 | 0.7 | 1.1 | 8.2 | 12.3 | 0.0 | 0.0 | 3.2 | 4.8 | 3.1 | 4.7 | 4.9 | 7.4 |
| Fri | 14.7 | 22.0 | 6.4 | 9.6 | 1.2 | 1.7 | 25.5 | 38.3 | 1.3 | 2.0 | 3.7 | 5.5 | 5.5 | 8.3 | 6.0 | 9.0 |
| Sat | 45.8 | 68.7 | 19.0 | 28.5 | 4.3 | 6.4 | 36.9 | 55.4 | 1.0 | 1.5 | 10.2 | 15.2 | 16.6 | 24.9 | 25.4 | 38.1 |
| Sun | 40.6 | 61.0 | 15.7 | 23.5 | 4.6 | 6.9 | 34.6 | 51.9 | 1.7 | 2.5 | 24.8 | 37.2 | 57.5 | 86.2 | 32.3 | 48.5 |
| AV | 19.8 | 29.6 | 14.1 | 21.2 | 2.5 | 3.7 | 24.0 | 36.0 | 1.2 | 1.3 | 8.1 | 12.2 | 15.2 | 22.8 | 12.8 | 19.2 |
| SD | 16.4 | 24.6 | 7.9 | 11.8 | 1.5 | 2.3 | 10.7 | 16.1 | 0.3 | 1.0 | 7.9 | 11.8 | 19.5 | 29.3 | 11.3 | 17.0 |
| CV | 0.8 | 0.8 | 0.56 | 0.56 | 0.63 | 0.63 | 0.45 | 0.45 | 0.24 | 0.73 | 0.97 | 0.97 | 1.3 | 1.3 | 0.9 | 0.9 |

**(±)-MDMA**

| | **UK** | | **Norway** | | **Italy** | | **Netherlands** | | **Spain** | | **Belgium** | | **Switzerland** | | **Denmark** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | ESTIMATED CONSUMPTION |
| Mon | 50.7 | 76.0 | 69.3 | 103.9 | 7.3 | 10.9 | 44.5 | 66.8 | 3.3 | 5.0 | 27.6 | 41.4 | 57.5 | 86.2 | 32.8 | 49.2 |
| Tue | 25.4 | 38.1 | 29.6 | 44.4 | 2.8 | 4.3 | 86.8 | 130.2 | 2.9 | 4.3 | 10.8 | 16.3 | 20.0 | 30.0 | 18.2 | 27.3 |
| Wed | 22.9 | 34.3 | 24.7 | 37.0 | 3.0 | 4.6 | 51.3 | 76.9 | 0.0 | 0.0 | 9.7 | 14.5 | 10.3 | 15.5 | 11.6 | 17.5 |
| Thur | 16.3 | 24.4 | 30.3 | 45.4 | 1.4 | 2.1 | 19.4 | 29.1 | 0.0 | 0.0 | 8.8 | 13.2 | 8.6 | 13.0 | 12.5 | 18.8 |
| Fri | 34.9 | 52.4 | 17.7 | 26.5 | 3.5 | 5.2 | 62.9 | 94.3 | 3.3 | 4.9 | 10.3 | 15.5 | 14.2 | 21.3 | 15.0 | 22.6 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sat | 111.8 | 167.6 | 45.8 | 68.7 | 12.0 | 18.1 | 91.2 | 136.8 | 2.5 | 3.8 | 25.7 | 38.6 | 44.4 | 66.6 | 56.6 | 84.9 |
| Sun | 109.9 | 164.9 | 46.4 | 69.5 | 17.7 | 26.6 | 78.1 | 117.1 | 4.1 | 6.2 | 60.7 | 91.0 | 149.4 | 224.0 | 77.5 | 116.3 |
| AV | 53.1 | 79.7 | 37.7 | 56.5 | 6.8 | 10.3 | 62.0 | 93.0 | 3.2 | 3.5 | 21.9 | 32.9 | 43.5 | 65.2 | 32.0 | 48.1 |
| SD | 40.9 | 61.4 | 17.5 | 26.2 | 6.0 | 9.0 | 25.7 | 38.5 | 0.6 | 2.5 | 18.8 | 28.3 | 50.2 | 75.3 | 25.7 | 38.5 |
| CV | 0.8 | 0.8 | 0.46 | 0.46 | 0.88 | 0.88 | 0.41 | 0.41 | 0.18 | 0.71 | 0.86 | 0.86 | 1.2 | 1.2 | 0.8 | 0.8 |

**Table S11** MDA loads.

| **R-(-)-MDA** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 1.5 | 0.9 | - | 1.7 | - | - | - | - |
| Tuesday | 1.0 | - | - | 1.8 | - | - | - | - |
| Wednesday | - | - | - | 1.4 | - | - | - | - |
| Thursday | - | - | - | 0.8 | - | - | - | 0.6 |
| Friday | 0.6 | - | - | 0.8 | - | - | - | - |
| Saturday | 1.0 | - | - | 1.3 | - | - | - | - |
| Sunday | 2.1 | - | - | 1.7 | - | - | - | - |
| AV | 0.9 | 0.1 | 0.0 | 1.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 0.8 | 0.3 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 0.9 | 2.6 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| **S-(+)-MDA** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 1.6 | - | - | 2.4 | - | - | - | - |
| Tuesday | - | - | - | 2.1 | - | - | - | - |
| Wednesday | - | - | - | 0.6 | - | - | - | - |
| Thursday | - | - | - | 0.8 | - | - | - | 0.3 |
| Friday | - | - | - | 1.3 | - | 1.1 | - | - |
| Saturday | 3.2 | 0.4 | - | 1.9 | - | - | - | - |
| Sunday | 2.5 | 2.2 | - | 3.9 | - | - | - | - |
| AV | 1.1 | 0.4 | 0.0 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 1.4 | 0.8 | 0.0 | 1.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 1.3 | 2.2 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 |
| **(±)-MDA** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 3.2 | 0.9 | - | 4.1 | - | - | - | - |
| Tuesday | 1.0 | - | - | 4.0 | - | - | - | - |
| Wednesday | - | - | - | 1.9 | - | - | - | - |
| Thursday | - | - | - | 1.6 | - | - | - | 0.9 |
| Friday | 0.6 | - | - | 2.1 | - | 1.1 | - | - |
| Saturday | 4.2 | 0.4 | - | 3.2 | - | - | - | - |
| Sunday | 4.7 | 2.2 | - | 5.7 | - | - | - | - |
| AV | 1.9 | 0.5 | 0.0 | 3.2 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 2.0 | 0.8 | 0.0 | 1.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 1.0 | 1.6 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 |

**Table S12** HMA loads.

| **E1-HMA** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 17.6 | - | - | - | - | - | - | - |
| Tuesday | - | - | - | - | - | - | - | - |
| Wednesday | - | - | - | - | - | - | - | - |
| Thursday | - | - | - | - | - | - | - | - |
| Friday | - | - | - | - | - | - | - | - |
| Saturday | - | - | - | 6.0 | - | - | - | - |
| Sunday | 15.3 | - | - | - | - | - | - | - |
| AV | 4.7 | 0.1 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 8.0 | 0.3 | 0.0 | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 1.7 | 2.6 | 0.0 | 2.6 | 0.0 | 0.0 | 0.0 | 0.0 |
| **E2-HMA** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | - | - | - | 6.4 | - | - | - | - |
| Tuesday | - | - | - | - | - | - | - | - |
| Wednesday | - | - | - | - | - | - | - | - |
| Thursday | - | - | - | - | - | - | - | - |
| Friday | - | - | - | - | - | - | - | - |
| Saturday | - | - | - | 5.7 | - | - | - | - |
| Sunday | 18.7 | - | - | 5.9 | - | - | - | - |
| AV | 2.7 | 0.1 | 0.0 | 2.6 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 7.1 | 0.2 | 0.0 | 3.2 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 2.6 | 1.8 | 0.0 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| **(±)-HMA** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 17.6 | - | - | 6.4 | - | - | - | - |
| Tuesday | - | - | - | - | - | - | - | - |
| Wednesday | - | - | - | - | - | - | - | - |
| Thursday | - | - | - | - | - | - | - | - |
| Friday | - | - | - | - | - | - | - | - |
| Saturday | - | - | - | 11.7 | - | - | - | - |
| Sunday | 34.0 | - | - | 5.9 | - | - | - | - |
| AV | 7.4 | 0.2 | 0.0 | 3.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 13.4 | 0.3 | 0.0 | 4.7 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 1.8 | 1.5 | 0.0 | 1.4 | 0.0 | 0.0 | 0.0 | 0.0 |

**Table S13** HMMA loads.

| **E1-HMMA** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 8.5 | - | - | - | 3.8 | 9.1 | 8.2 | 5.3 |
| Tuesday | 8.6 | - | - | - | 8.2 | 6.5 | - | - |
| Wednesday | 8.3 | - | - | 6.0 | - | 4.2 | - | - |
| Thursday | - | - | - | 5.8 | - | 4.5 | - | 5.0 |
| Friday | 10.5 | - | - | 7.0 | - | 9.0 | - | 4.2 |
| Saturday | 9.6 | - | - | 6.4 | - | 9.2 | 9.0 | 4.9 |
| Sunday | 8.5 | - | - | 6.4 | 3.8 | 9.3 | 11.2 | 4.9 |
| AV | 7.7 | 0.1 | 0.0 | 4.5 | 2.3 | 7.4 | 4.1 | 3.5 |
| SD | 3.5 | 0.3 | 0.0 | 3.1 | 3.2 | 2.3 | 5.1 | 2.4 |
| CV | 0.5 | 2.6 | 0.0 | 0.7 | 1.4 | 0.3 | 1.3 | 0.7 |
| **E2-HMMA** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 13.0 | - | - | 9.3 | 12.0 | 7.1 | 6.3 | 8.1 |
| Tuesday | - | - | - | 8.9 | 12.9 | 5.4 | - | - |
| Wednesday | - | - | - | 9.3 | - | 1.9 | - | - |
| Thursday | - | - | - | 8.7 | - | 7.1 | - | - |
| Friday | 13.5 | - | - | 9.6 | - | 14.2 | - | 7.9 |
| Saturday | 14.8 | - | - | 9.1 | - | 14.3 | 14.2 | 7.6 |
| Sunday | 13.0 | - | - | 9.8 | 11.9 | 14.2 | 17.5 | 7.6 |
| AV | 7.8 | 0.1 | 0.0 | 9.2 | 5.3 | 9.2 | 5.4 | 4.4 |
| SD | 7.3 | 0.2 | 0.0 | 0.4 | 6.6 | 5.0 | 7.5 | 4.2 |
| CV | 0.9 | 1.8 | 0.0 | 0.0 | 1.2 | 0.5 | 1.4 | 0.9 |
| **(±)-HMMA** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 21.4 | - | - | 9.3 | 15.8 | 16.3 | 14.5 | 13.4 |
| Tuesday | 8.6 | - | - | 8.9 | 21.1 | 12.0 | - | - |
| Wednesday | 8.3 | - | - | 15.4 | - | 6.1 | - | - |
| Thursday | - | - | - | 14.5 | - | 11.6 | - | 5.0 |
| Friday | 24.0 | - | - | 16.6 | - | 23.2 | - | 12.1 |
| Saturday | 24.4 | - | - | 15.5 | - | 23.5 | 23.2 | 12.5 |
| Sunday | 21.5 | - | - | 16.1 | 15.7 | 23.5 | 28.7 | 12.5 |
| AV | 15.5 | 0.2 | 0.0 | 13.8 | 7.5 | 16.6 | 9.5 | 7.9 |
| SD | 9.7 | 0.3 | 0.0 | 3.2 | 9.5 | 7.0 | 12.5 | 6.1 |
| CV | 0.6 | 1.5 | 0.0 | 0.2 | 1.3 | 0.4 | 1.3 | 0.8 |

**Table S14** Mephedrone loads.

| **R-(+)-Mephedrone** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 14.9 | - | - | - | - | - | - | - |
| Tuesday | 8.5 | - | - | - | - | - | - | - |
| Wednesday | 8.5 | - | - | - | - | - | - | - |
| Thursday | 8.2 | - | - | - | - | - | - | - |
| Friday | 12.7 | - | - | - | - | - | - | - |
| Saturday | 26.3 | - | - | - | - | - | - | - |
| Sunday | 19.6 | - | - | - | - | - | - | - |
| AV | 14.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 6.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| **S-(-)-Mephedrone** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 16.1 | - | - | - | - | - | - | - |
| Tuesday | 6.3 | - | - | - | - | - | - | - |
| Wednesday | 8.5 | - | - | - | - | - | - | - |
| Thursday | 7.4 | - | - | - | - | - | - | - |
| Friday | 8.1 | - | - | - | - | - | - | - |
| Saturday | 21.4 | - | - | - | - | - | - | - |
| Sunday | 12.5 | - | - | - | - | - | - | - |
| AV | 11.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 5.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| **(±)-Mephedrone** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 31.1 | - | - | - | - | - | - | - |
| Tuesday | 14.9 | - | - | - | - | - | - | - |
| Wednesday | 17.1 | - | - | - | - | - | - | - |
| Thursday | 15.6 | - | - | - | - | - | - | - |
| Friday | 20.8 | - | - | - | - | - | - | - |
| Saturday | 47.7 | - | - | - | - | - | - | - |
| Sunday | 32.1 | - | - | - | - | - | - | - |
| AV | 25.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| SD | 12.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| CV | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

**Table S15** Norephedrine loads.

| **E1-Norephedrine** | | | | |
|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** |
| Monday | - | 25.0 | 2.7 | - |
| Tuesday | - | 23.1 | 4.6 | -- |
| Wednesday | - | 37.3 | 3.7 | -- |
| Thursday | - | 27.6 | 3.0 | -- |
| Friday | - | 18.6 | 2.6 | -- |
| Saturday | - | 22.6 | 4.4 | -- |
| Sunday | - | 22.9 | 2.3 | -- |
| AV | 0.0 | 25.3 | 3.3 | 0.0 |
| SD | 0.0 | 6.0 | 0.9 | 0.0 |
| CV | 0.0 | 0.2 | 0.3 | 0.0 |
| **E2-Norephedrine** | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** |
| Monday | - | 30.3 | 3.8 | - |
| Tuesday | - | 18.3 | 2.3 | - |
| Wednesday | 7.4 | 33.9 | 4.0 | - |
| Thursday | 2.5 | 24.4 | 4.1 | - |
| Friday | 5.1 | 21.0 | 5.1 | - |
| Saturday | 5.3 | 26.1 | 4.0 | - |
| Sunday | 3.6 | 25.9 | 3.1 | - |
| AV | 3.4 | 25.7 | 3.8 | 0.0 |
| SD | 2.8 | 5.3 | 0.9 | 0.0 |
| CV | 0.8 | 0.2 | 0.2 | 0.0 |
| **(±)-Norephedrine** | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** |
| Monday | - | 55.2 | 6.5 | - |
| Tuesday | - | 41.3 | 6.9 | - |
| Wednesday | 7.4 | 71.2 | 7.7 | - |
| Thursday | 2.5 | 52.0 | 7.1 | - |
| Friday | 5.1 | 39.7 | 7.7 | - |
| Saturday | 5.3 | 48.6 | 8.4 | - |
| Sunday | 3.6 | 48.7 | 5.4 | - |
| AV | 3.4 | 51.0 | 7.1 | 0.0 |
| SD | 2.8 | 10.5 | 1.0 | 0.0 |
| CV | 0.8 | 0.2 | 0.1 | 0.0 |

**Table S16** Ephedrines loads.

| **1R,2S-(-)-Ephedrine** | | | |
|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | |
| | **UK** | **Norway** | **Italy** |
| Monday | - | 0.4 | 0.5 |
| Tuesday | 1.8 | - | 3.8 |
| Wednesday | 0.3 | - | 0.7 |
| Thursday | - | 1.6 | 4.5 |
| Friday | 0.4 | 1.4 | - |
| Saturday | 1.2 | 0.0 | 14.3 |
| Sunday | - | 1.3 | - |
| AV | 0.6 | 0.7 | 3.4 |
| SD | 0.7 | 0.7 | 5.1 |
| CV | 1.3 | 1.1 | 1.5 |
| **1S,2S-(+)-Pseudoephedrine** | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | |
| | **UK** | **Norway** | **Italy** |
| Monday | 126.7 | 21.9 | 36.4 |
| Tuesday | 81.2 | 20.9 | 39.6 |
| Wednesday | 98.4 | 24.1 | 36.7 |
| Thursday | 55.7 | 20.7 | 43.5 |
| Friday | 134.1 | 24.8 | 30.4 |
| Saturday | 89.2 | 18.6 | 30.4 |
| Sunday | 89.4 | 17.2 | 33.0 |
| AV | 96.4 | 21.2 | 35.7 |
| SD | 26.9 | 2.7 | 4.8 |
| CV | 0.3 | 0.1 | 0.1 |

**Table S17** Venlafaxine loads.

| **E1-Venlafaxine** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 21.7 | 25.0 | 11.9 | 22.8 | 45.6 | 97.7 | 57.0 | 46.2 |
| Tuesday | 19.6 | 31.8 | 13.5 | 25.0 | 47.7 | 113.9 | 48.3 | 53.7 |
| Wednesday | 20.1 | 26.4 | 12.5 | 28.2 | 30.9 | 47.9 | 52.9 | 45.5 |
| Thursday | 20.6 | 21.8 | 11.6 | 30.3 | 70.5 | 117.2 | 54.6 | 49.4 |
| Friday | 17.4 | 21.0 | 18.6 | 28.6 | 50.3 | 127.5 | 57.1 | 52.5 |
| Saturday | 19.7 | 37.6 | 13.9 | 24.1 | 41.1 | 127.5 | 56.1 | 54.8 |
| Sunday | 17.4 | 27.2 | 15.4 | 26.0 | 48.2 | 106.0 | 55.1 | 42.9 |
| AV | 19.5 | 27.2 | 13.9 | 26.4 | 47.8 | 105.4 | 54.4 | 49.3 |
| SD | 1.6 | 5.8 | 2.4 | 2.7 | 12.0 | 27.6 | 3.1 | 4.6 |
| CV | 0.1 | 0.2 | 0.2 | 0.1 | 0.3 | 0.3 | 0.1 | 0.1 |
| **E2-Venlafaxine** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 23.9 | 26.7 | 14.7 | 25.3 | 41.5 | 88.6 | 54.9 | 49.0 |
| Tuesday | 22.8 | 36.6 | 13.1 | 25.3 | 41.0 | 106.4 | 47.9 | 49.2 |
| Wednesday | 22.3 | 36.2 | 14.7 | 28.3 | 28.1 | 52.3 | 50.2 | 43.8 |
| Thursday | 19.4 | 25.5 | 11.6 | 37.5 | 64.5 | 116.5 | 54.2 | 48.6 |
| Friday | 21.5 | 26.3 | 15.0 | 28.6 | 44.0 | 124.1 | 57.5 | 55.0 |
| Saturday | 22.6 | 39.4 | 16.1 | 25.8 | 36.0 | 121.9 | 53.7 | 58.4 |
| Sunday | 20.8 | 29.3 | 12.7 | 27.2 | 42.5 | 96.1 | 56.3 | 48.1 |
| AV | 21.9 | 31.4 | 14.0 | 28.3 | 42.5 | 100.8 | 53.5 | 50.3 |
| SD | 1.5 | 5.8 | 1.6 | 4.3 | 11.1 | 25.1 | 3.4 | 4.8 |
| CV | 0.1 | 0.2 | 0.1 | 0.2 | 0.3 | 0.2 | 0.1 | 0.1 |
| **(±)-Venlafaxine** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 45.7 | 51.7 | 26.6 | 48.1 | 87.1 | 186.3 | 111.9 | 95.2 |
| Tuesday | 42.4 | 68.3 | 26.5 | 50.2 | 88.7 | 220.3 | 96.2 | 102.9 |
| Wednesday | 42.4 | 62.6 | 27.1 | 56.5 | 58.9 | 100.2 | 103.1 | 89.2 |
| Thursday | 40.0 | 47.3 | 23.2 | 67.8 | 134.9 | 233.7 | 108.8 | 97.9 |
| Friday | 38.9 | 47.3 | 33.6 | 57.1 | 94.4 | 251.6 | 114.6 | 107.5 |
| Saturday | 42.3 | 76.9 | 30.0 | 49.9 | 77.2 | 249.5 | 109.8 | 113.1 |
| Sunday | 38.2 | 56.5 | 28.1 | 53.2 | 90.7 | 202.1 | 111.4 | 91.0 |
| AV | 41.4 | 58.7 | 27.9 | 54.7 | 90.3 | 206.3 | 108.0 | 99.6 |
| SD | 2.6 | 11.2 | 3.3 | 6.7 | 23.0 | 52.5 | 6.3 | 8.8 |
| CV | 0.1 | 0.2 | 0.1 | 0.1 | 0.3 | 0.3 | 0.1 | 0.1 |

**Table S18** Desmethylvenlafaxine loads.

| **E1-Desmethylvenlafaxine** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 56.9 | 54.4 | 20.6 | 60.8 | 137.9 | 182.8 | 118.8 | 131.4 |
| Tuesday | 60.9 | 75.7 | 48.8 | 60.6 | 152.5 | 211.3 | 114.6 | 150.1 |
| Wednesday | 56.1 | 82.1 | 44.7 | 56.8 | 110.1 | 120.1 | 116.9 | 123.6 |
| Thursday | 54.6 | 52.0 | 36.0 | 59.3 | 179.0 | 247.0 | 124.1 | 142.1 |
| Friday | 42.9 | 55.4 | 57.4 | 62.7 | 163.4 | 232.9 | 105.1 | 143.3 |
| Saturday | 52.1 | 54.8 | 28.2 | 56.8 | 130.6 | 246.3 | 108.8 | 139.9 |
| Sunday | 51.1 | 46.6 | 28.1 | 57.5 | 155.4 | 198.5 | 110.2 | 121.5 |
| AV | 53.5 | 60.2 | 37.7 | 59.2 | 147.0 | 205.6 | 114.1 | 136.0 |
| SD | 5.7 | 13.3 | 13.2 | 2.3 | 22.8 | 44.8 | 6.5 | 10.7 |
| CV | 0.1 | 0.2 | 0.3 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 |
| **E2-Desmethylvenlafaxine** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 29.5 | 35.1 | 14.1 | 34.3 | 87.5 | 75.3 | 73.9 | 84.0 |
| Tuesday | 33.1 | 49.2 | 25.8 | 33.7 | 96.8 | 98.1 | 68.3 | 93.5 |
| Wednesday | 30.9 | 52.3 | 22.4 | 33.6 | 67.8 | 52.5 | 73.6 | 76.5 |
| Thursday | 27.3 | 38.8 | 25.1 | 32.3 | 107.5 | 110.8 | 76.2 | 85.1 |
| Friday | 24.9 | 31.1 | 36.6 | 36.0 | 94.9 | 104.2 | 67.3 | 92.1 |
| Saturday | 30.1 | 27.0 | 20.5 | 33.4 | 77.8 | 113.6 | 72.2 | 92.1 |
| Sunday | 25.7 | 35.4 | 13.8 | 33.0 | 89.9 | 90.3 | 67.6 | 76.0 |
| AV | 28.8 | 38.4 | 22.6 | 33.8 | 88.9 | 92.1 | 71.3 | 85.6 |
| SD | 2.9 | 9.2 | 7.8 | 1.2 | 13.0 | 21.8 | 3.5 | 7.4 |
| CV | 0.1 | 0.2 | 0.3 | 0.0 | 0.1 | 0.2 | 0.0 | 0.1 |
| **(±)-Desmethylvenlafaxine** | | | | | | | | |
| | **POPULATION-NORMALISED MASS LOADS (mg/1000 people/day)** | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 86.4 | 89.4 | 34.8 | 95.2 | 225.4 | 258.2 | 192.7 | 215.4 |
| Tuesday | 94.0 | 124.9 | 74.6 | 94.4 | 249.3 | 309.4 | 182.9 | 243.6 |
| Wednesday | 86.9 | 134.4 | 67.1 | 90.4 | 177.9 | 172.6 | 190.4 | 200.1 |
| Thursday | 81.9 | 90.8 | 61.1 | 91.6 | 286.5 | 357.9 | 200.3 | 227.2 |
| Friday | 67.8 | 86.5 | 94.0 | 98.7 | 258.2 | 337.1 | 172.5 | 235.4 |
| Saturday | 82.3 | 81.8 | 48.7 | 90.2 | 208.4 | 359.9 | 181.0 | 232.0 |
| Sunday | 76.8 | 81.9 | 41.9 | 90.5 | 245.3 | 288.8 | 177.8 | 197.5 |
| AV | 82.3 | 98.5 | 60.3 | 93.0 | 235.9 | 297.7 | 185.4 | 221.6 |
| SD | 8.3 | 21.7 | 20.5 | 3.2 | 35.5 | 66.4 | 9.6 | 17.8 |
| CV | 0.1 | 0.2 | 0.3 | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 |

**Table S19** Tramadol loads.

| D1-Tramadol | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 128.6 | 67.5 | 15.8 | 45.5 | 104.9 | 377.3 | 72.6 | 162.4 |
| Tuesday | 118.7 | 76.2 | 20.4 | 44.0 | 118.7 | 342.2 | 66.3 | 188.7 |
| Wednesday | 130.0 | 95.3 | 22.4 | 41.9 | 86.7 | 303.3 | 71.6 | 155.2 |
| Thursday | 126.9 | 64.2 | 21.7 | 38.1 | 129.5 | 385.0 | 75.0 | 184.4 |
| Friday | 110.6 | 64.5 | 31.8 | 39.1 | 121.5 | 367.1 | 65.8 | 178.0 |
| Saturday | 119.2 | 59.7 | 18.3 | 41.1 | 97.9 | 891.6 | 77.1 | 188.4 |
| Sunday | 109.5 | 56.5 | 21.1 | 44.9 | 110.7 | 419.2 | 85.9 | 154.3 |
| AV | 120.5 | 69.1 | 21.6 | 42.1 | 110.0 | 440.8 | 73.5 | 173.1 |
| SD | 8.4 | 13.1 | 5.0 | 2.8 | 14.7 | 202.0 | 6.9 | 15.4 |
| CV | 0.1 | 0.2 | 0.2 | 0.1 | 0.1 | 0.5 | 0.1 | 0.1 |
| **D2-Tramadol** | | | | | | | | |
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 104.5 | 45.6 | 13.6 | 35.2 | 72.8 | 202.8 | 57.9 | 127.2 |
| Tuesday | 108.2 | 54.8 | 14.6 | 33.1 | 79.3 | 198.5 | 48.7 | 136.2 |
| Wednesday | 111.3 | 76.4 | 18.7 | 37.2 | 64.2 | 171.9 | 55.2 | 122.0 |
| Thursday | 98.4 | 51.0 | 16.9 | 31.4 | 93.3 | 221.1 | 57.3 | 135.6 |
| Friday | 105.4 | 48.7 | 18.6 | 32.0 | 85.2 | 225.5 | 52.0 | 131.0 |
| Saturday | 111.6 | 45.1 | 14.7 | 30.5 | 64.0 | 477.9 | 56.6 | 147.7 |
| Sunday | 98.3 | 51.3 | 15.0 | 34.2 | 72.6 | 224.8 | 64.6 | 124.8 |
| AV | 105.4 | 53.3 | 16.0 | 33.4 | 75.9 | 246.1 | 56.0 | 132.0 |
| SD | 5.5 | 10.7 | 2.1 | 2.3 | 10.8 | 104.0 | 5.0 | 8.7 |
| CV | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 | 0.4 | 0.1 | 0.1 |
| **(±)-Tramadol** | | | | | | | | |
| | POPULATION-NORMALISED MASS LOADS (mg/1000 people/day) | | | | | | | |
| | **UK** | **Norway** | **Italy** | **Netherlands** | **Spain** | **Belgium** | **Switzerland** | **Denmark** |
| Monday | 233.1 | 113.1 | 29.3 | 80.8 | 177.7 | 580.0 | 130.5 | 289.6 |
| Tuesday | 226.8 | 131.0 | 35.0 | 77.1 | 198.0 | 540.7 | 115.0 | 324.9 |
| Wednesday | 241.3 | 171.7 | 41.1 | 79.1 | 150.9 | 475.2 | 126.8 | 277.2 |
| Thursday | 225.2 | 115.2 | 38.6 | 69.5 | 222.8 | 606.1 | 132.3 | 320.0 |
| Friday | 216.0 | 113.2 | 50.5 | 71.2 | 206.7 | 592.7 | 117.7 | 309.0 |
| Saturday | 230.9 | 104.8 | 33.0 | 71.7 | 161.9 | 1369.5 | 133.7 | 336.0 |
| Sunday | 207.8 | 107.8 | 36.1 | 79.1 | 183.2 | 644.0 | 150.5 | 279.1 |
| AV | 225.9 | 122.4 | 37.7 | 75.5 | 185.9 | 686.9 | 129.5 | 305.1 |
| SD | 11.1 | 23.3 | 6.8 | 4.6 | 25.2 | 305.7 | 11.7 | 23.4 |
| CV | 0.0 | 0.2 | 0.2 | 0.1 | 0.1 | 0.4 | 0.1 | 0.1 |

**S1-Statistical tests.**

Wastewater samples from 2015 sampling campaign: amphetamine.

F-Test Two-Sample for Variances

| | *EF* | *EF of standards* |
|---|---|---|
| Mean | 0.460782672 | 0.516421 |
| Variance | 0.000181069 | 0.0001 |
| Observations | 5 | 7 |
| df | 4 | 6 |
| F | 1.804179405 | |
| P(F<=f) one-tail | 0.246815412 | |
| F Critical one-tail | 4.53367695 | |

Data:

| Amphetamine 2015 (no Switzerland) | Validation |
|---|---|
| EF | EF of standards |
| 0.45 | 0.53 |
| 0.47 | 0.52 |
| 0.45 | 0.53 |
| 0.48 | 0.51 |
| 0.45 | 0.50 |
| - | 0.51 |
| - | 0.52 |

$\alpha$=0.05

| t-Test: Two-Sample Assuming Equal Variances | | |
|---|---|---|
| | EF of standards from validation | EF |
| Mean | 0.516421 | 0.460783 |
| Variance | 0.0001 | 0.000181 |
| Observations | 7 | 5 |
| Pooled Variance | 0.000133 | |
| Hypothesized Mean Difference | 0 | |
| df | 10 | |
| t Stat | 8.250336 | |
| P(T<=t) one-tail | 4.49E-06 | |
| t Critical one-tail | 1.812461 | |
| P(T<=t) two-tail | 0.0000090 | |

$\alpha$=0.001

| t-Test: Two-Sample Assuming Equal Variances | | |
|---|---|---|
| t Stat | 8.250336 | |
| P(T<=t) one-tail | 4.49E-06 | |
| t Critical one-tail | 4.1437 | |
| P(T<=t) two-tail | 8.98E-06 | |
| t Critical two-tail | 4.586894 | |

Moffat, A. C., D. M. Osselton and Widdop (2004). Clarke's analysis of drugs and poisons, Pharmaceutical press

# Appendix 4

The following supplementary data are contained in Appendix 4:

Table S1 Selected analytes and their properties (MW=molecular weight).

Table S2 Studied mobile phase compositions with CHIRALCEL® OZ-RH column.

Table S3 Validation parameters -instrumental precision

Figure S1 CHIRALCEL® OZ-RH column – impact of the organic content on the separation of studied analytes (mobile phases in the legend are referred to the organic modifier mentioned in the title of the graphic).

Figure S2 CHIRALCEL® OZ-RH column – (a) impact of the organic modifiers used on the separation of studied analytes. The mobile phase composition was constituted by the organic solvent specified in the legend and by 5% of 5mM ammonium acetate as aqueous content; (b) Impact of the nature of the organic content in the mobile phase on enantiomeric resolution. The mobile phases considered were the best performing: 95:5 5mM ammonium formate for IPA and

ACN, 99:1 5mM ammonium formate for EtOH, 99:1 10mM ammonium formate for MeOH.

Figure S3 CHIRALCEL® OZ-RH column – impact of (a) the flow rate, (b) pH and (c) percentage of the additive content on the separation of studied analytes. The mobile phase used for the first graphic was made of acetonitrile:water 95:5 5mM ammonium acetate.

**Table S1** Selected analytes and their properties (MW=molecular weight).

| Compound | CAS | Formula | MW | LogP | $pK_a$ | | Supplier |
|---|---|---|---|---|---|---|---|
| | | | | | Strongest acidic | Strongest basic | |
| Ciprofloxacin | 85721-33-1 | $C_{17}H_{18}FN_3O_3$ | 331.3 | -0.81[a] | 5.76[a] | 8.68[a] | Fluka |
| Desethylene-ciprofloxacin | 528851-31-2 | $C_{15}H_{17}ClFN_3O_3$ | 341.8 | - | - | - | TRC |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 100986-85-4 | $C_{18}H_{20}FN_3O_4$ | | 0.65[a] | 5.45[a] | 6.20[a] | Sigma Aldrich |
| (±)-Ofloxacin | 82419-36-1 | $C_{18}H_{20}FN_3O_4$ | 361.4 | 0.65[a] | 5.45[a] | 6.20[a] | Sigma Aldrich |
| Norfloxacin | 70458-96-7 | $C_{16}H_{18}FN_3O_3$ | 319.3 | -0.92[a] | 5.77[a] | 8.68[a] | Fluka |
| (±)-Ofloxacin-*N*-oxide | 104721-52-0 | $C_{20}H_{24}FN_3O_7$ | 437.4 | - | - | - | TRC |
| (±)-Desmethyl-ofloxacin | 82419-52-1 | $C_{17}H_{18}FN_3O_4$ | 347.3 | - | - | - | TRC |
| Nalidixic acid | 3374-05-8 | $C_{12}H_{11}N_2NaO_3$ | 254.2 | 1.01[a] | 5.95[a] | 4.68[a] | Sigma Aldrich |
| (±)-Lomefloxacin | 98079-52-8 | $C_{17}H_{19}F_2N_3O_3$ | | -0.30[e] | 5.64[e] | 8.70[e] | Sigma Aldrich |
| *R,R*-Moxifloxacin | 1346603-25-5 | $C_{21}H_{25}ClFN_3O_4$ | 437.9 | 2.9[e] | 5.69[a] | 9.42[a] | TRC |
| *S,S*- Moxifloxacin | 192927-63-2 | $C_{21}H_{27}ClFN_3O_5$ | 455.9 | 2.9[e] | 5.69[a] | 9.42[a] | TRC |
| Moxifloxacin-*N*-sulphate | n.a. | $C_{21}H_{22}FN_3Na_2O_7S$ | 525.5 | - | - | - | TRC |
| (±)-Prulifloxacin | 123447-62-1 | $C_{21}H_{20}FN_3O_6S$ | 461.5 | 2.49[b] 3.27[c] | 5.85[d] | 6.25[d] | Sigma Aldrich |
| (±)-Ulifloxacin | 112984-60-8 | $C_{16}H_{16}FN_3O_3S$ | 349.4 | -0.56[d] | 5.85[d] | 8.69[d] | TRC |
| (±)-*cis*-Ketoconazole | 65277-42-1 | $C_{26}H_{28}Cl_2N_4O_4$ | | 4.19 [a] | - | 6.75[e] | Sigma Aldrich |
| (±)-Flumequine | 42835-25-6 | $C_{14}H_{12}FNO_3$ | | 2.42[a] | 6.00[e] | -4.30[e] | Sigma Aldrich |
| (±)-Nadifloxacin | 124858-35-1 | $C_{19}H_{21}FN_2O_4$ | 360.4 | 1.87[d] | 5.55[d] | 1.27[d] | TRC |
| *R*-(+)-Besifloxacin | 405165-61-9 | $C_{19}H_{22}Cl_2FN_3O_3$ | 430.3 | 0.54[a] | 5.64[e] | 9.67[e] | TRC |
| **Internal Standard** | | | | | | | |
| Ciprofloxacin $-D_8$ | | | | | | | QMX laboratories |
| (±)-Ofloxacin-$D_3$ | | | | | | | QMX laboratories |
| (±)-Desmethyl-ofloxacin-$D_8$ | | | | | | | TRC |
| (±)-Flumequine-$^{13}C_3$ | | | | | | | TRC |

n.a.-not available

[a] ChemAxon

[b] Chemicalize.org

[c] ChemSpider

[d] ChEMBL (www.ebi.ac.uk/chembldb)

[e] DrugBank

**Table S2** Studied mobile phase compositions with CHIRALCEL® OZ-RH column.

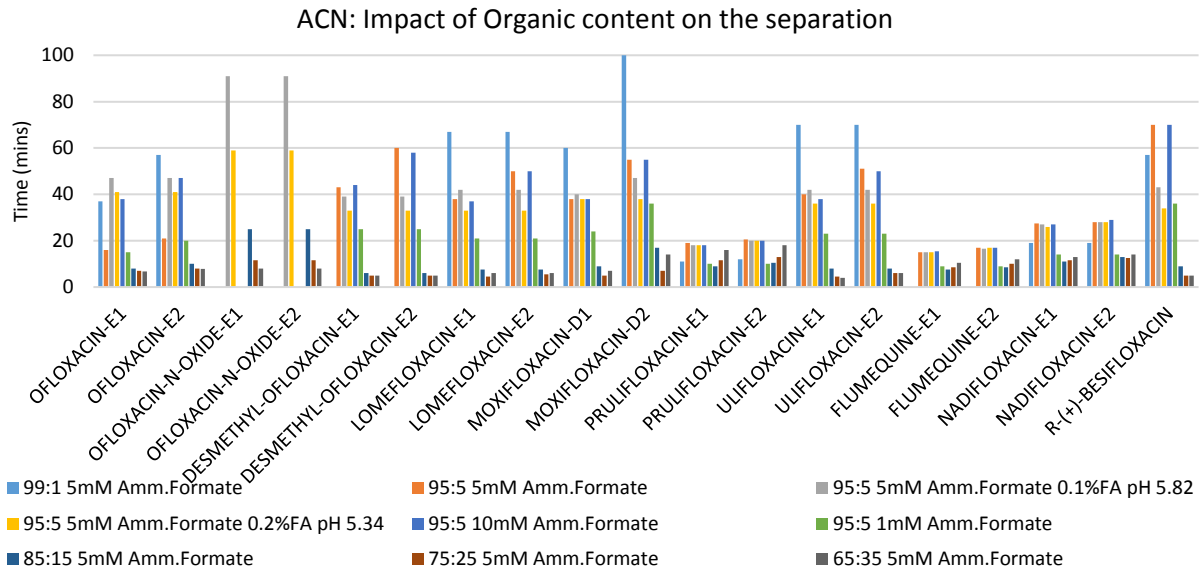
| **% ACN** | **% $H_2O$** | **Amm.Form. (mM)** | **pH** | **Flow rate (ml $min^{-1}$)** |
|---|---|---|---|---|
| 95 | 5 | 5 | 7.48 | 0.1 |
| 95 | 5 | 5 | 7.48 | 0.15 |
| 95 | 5 | 5 | 7.48 | 0.18 |
| 95 | 5 | 5 | 7.48 | 0.2 |
| 95 | 5 | 5+0.1%FA | 5.82 | 0.05 |
| 95 | 5 | 5+0.2%FA | 5.34 | 0.05 |
| 95 | 5 | 10 | 7.03 | 0.05 |
| 95 | 5 | 1 | 7.41 | 0.05 |
| 95 | 5 | 1 | 7.41 | 0.1 |
| 99 | 1 | 5 | 7.49 | 0.1 |
| 85 | 15 | 5 | 7.06 | 0.1 |
| 75 | 25 | 5 | 6.47 | 0.1 |
| 65 | 35 | 5 | 6.88 | 0.1 |
| **% EtOH** | **% $H_2O$** | **Amm.Form. (mM)** | **pH** | **Flow rate (ml $min^{-1}$)** |
| 99 | 1 | 5 | 7.51 | 0.1 |
| 95 | 5 | 5 | 7.39 | 0.1 |
| 95 | 5 | 10 | 7.49 | 0.1 |
| 85 | 15 | 5 | 7.09 | 0.1 |
| 85 | 15 | 5 | 4.82 | 0.1 |
| 75 | 25 | 5 | 7.04 | 0.1 |
| **% MeOH** | **% $H_2O$** | **Amm.Form. (mM)** | **pH** | **Flow rate (ml $min^{-1}$)** |
| 99 | 1 | 10+ 0.05%FA | 5.50 | 0.1 |
| 99 | 1 | 10 | 7.27 | 0.1 |
| 99 | 1 | 5 | 7.24 | 0.1 |
| 95 | 5 | 10+ 0.05%FA | 5.38 | 0.1 |
| 95 | 5 | 5+ 0.05%FA | 5.14 | 0.1 |
| 95 | 5 | 5 | 7.21 | 0.1 |
| 85 | 15 | 5 | 7.04 | 0.1 |
| 75 | 25 | 5 | 6.74 | 0.1 |
| 65 | 35 | 5 | 6.6 | 0.1 |
| **%IPA** | **% $H_2O$** | **Amm.Form. (mM)** | **pH** | **Flow rate (ml $min^{-1}$)** |
| 95 | 5 | 5 | 7.63 | 0.1 |
| **% ACN/MeOH** | **% $H_2O$** | **Amm.Form. (mM)** | **pH** | **Flow rate (ml $min^{-1}$)** |
| 95 | 5 | 5 | 7.37 | 0.1 |
| 90 | 10 | 5 | 7.2 | 0.1 |
| 80 | 20 | 5 | 7.12 | 0.1 |
| **% EtOH/MeOH** | **% $H_2O$** | **Amm.Form. (mM)** | **pH** | **Flow rate (ml $min^{-1}$)** |
| 95 | 5 | 5 | 7.42 | 0.1 |

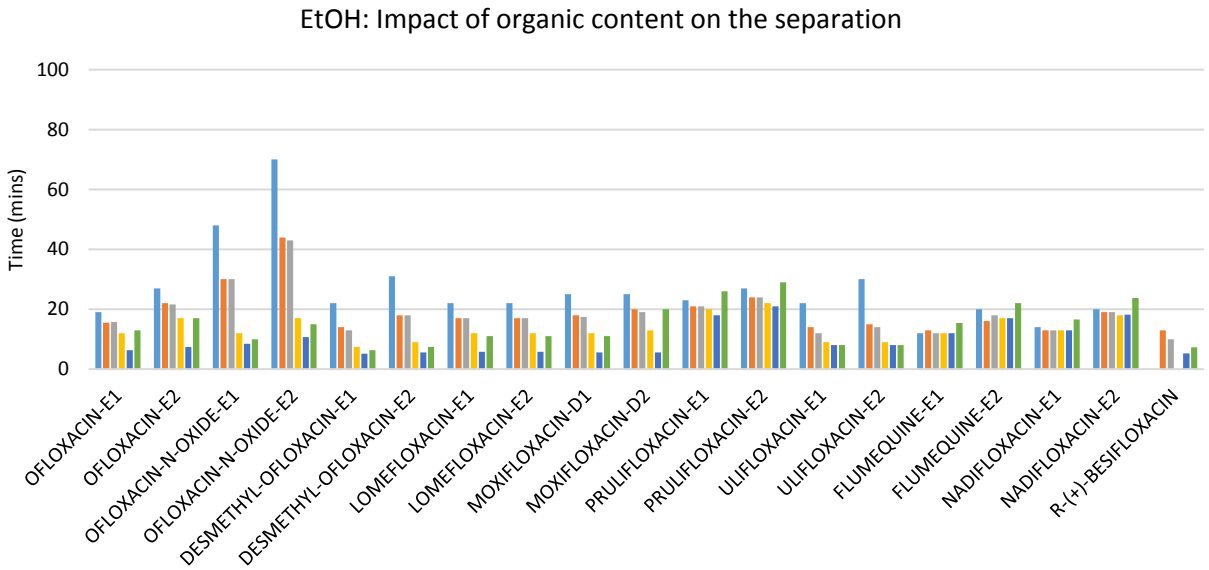
**Table S3** Validation parameters -instrumental precision

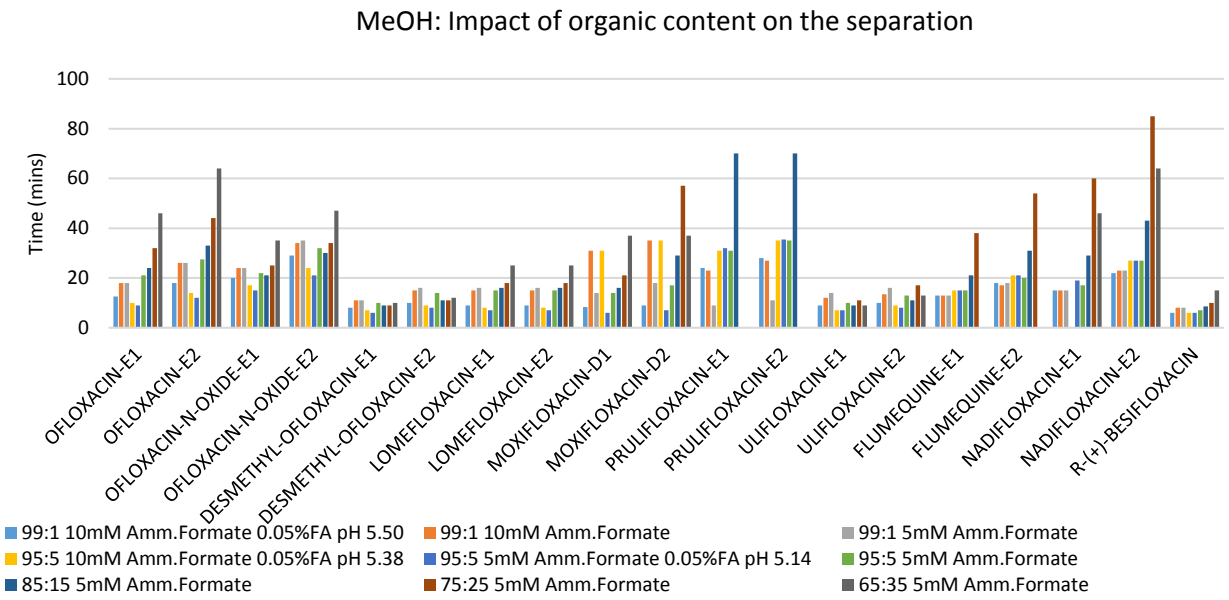
| | Intra-day RSD% (n=4) | | | | | | | | | Inter-day RSD% (n=3) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 µg/L** D 1* | 5 µg/L D 2 | 5 µg/L D 3 | 50 µg/L D 1 | 50 µg/L D 2 | 50 µg/L D 3 | 500 µg/L D 1 | 500 µg/L D 2 | 500 µg/L D 3 | 5 µg/L | 50 µg/L | 500 µg/L |
| Ciprofloxacin | 5.3 | 6.4 | 13.4 | 0.8 | 1.0 | 3.5 | 7.9 | 1.0 | 5.6 | 8.4 | 1.8 | 4.8 |
| Desethylene-ciprofloxacin | 5.4 | 6.0 | 17.6 | 10.9 | 14.4 | 5.7 | 9.0 | 3.0 | 6.5 | 9.7 | 10.3 | 6.2 |
| *S*-(-)-Ofloxacin (*L*-Ofloxacin) | 6.8 | 13.0 | 3.0 | 3.7 | 3.0 | 4.4 | 2.4 | 3.8 | 0.3 | 7.6 | 3.7 | 2.2 |
| *R*-(+)-Ofloxacin | 4.6 | 2.3 | 10.9 | 2.8 | 2.1 | 5.4 | 5.9 | 2.4 | 2.6 | 5.9 | 3.4 | 3.6 |
| Norfloxacin | 4.6 | 15.8 | 19.8 | 11.6 | 2.9 | 4.0 | 14.4 | 3.5 | 2.3 | 13.4 | 6.2 | 6.7 |
| *S*-(-)-Ofloxacin-*N*-oxide | 3.3 | 4.0 | 3.7 | 4.6 | 1.1 | 9.0 | 4.7 | 3.2 | 2.2 | 3.7 | 4.9 | 3.4 |
| *R*-(+)-Ofloxacin-*N*-oxide | 5.2 | 7.1 | 19.3 | 15.2 | 10.3 | 12.9 | 5.2 | 3.7 | 1.9 | 10.5 | 12.8 | 3.6 |
| *S*-(-)-Desmethyl-ofloxacin | 1.1 | 8.1 | 4.8 | 0.9 | 1.0 | 4.3 | 1.0 | 1.6 | 2.9 | 4.7 | 2.1 | 1.8 |
| *R*-(+)-Desmethyl-ofloxacin | 5.9 | 8.2 | 18.9 | 1.6 | 9.0 | 5.0 | 3.2 | 3.4 | 1.6 | 11.0 | 5.2 | 2.7 |
| Nalidixic acid | 3.0 | 8.7 | 2.7 | 5.6 | 4.5 | 4.9 | 5.3 | 1.6 | 3.4 | 4.8 | 5.0 | 3.4 |
| Lomefloxacin | 7.0 | 15,5 | 3.2 | 2.9 | 4.4 | 1.7 | 3.3 | 3.3 | 5.0 | 5.1 | 3.0 | 3.9 |
| *R*,*R*-Moxifloxacin | 7.5 | 19.7 | 10.0 | 0.0 | 10.2 | 11.9 | 0.9 | 1.0 | 8.0 | 12.4 | 7.4 | 3.3 |
| *S*,*S*- Moxifloxacin | 13.2 | 15.3 | 7.3 | 4.1 | 4.7 | 3.3 | 2.7 | 2.7 | 5.9 | 11.9 | 4.0 | 3.8 |
| Moxifloxacin-*N*-sulphate | 4.4 | 5.4 | 17.3 | 12.3 | 5.8 | 8.3 | 4.5 | 9.6 | 3.5 | 9.0 | 8.8 | 5.9 |
| Prulifloxacin-E1 | 2.3 | 23.3 | 15.8 | 6.9 | 2.5 | 0.8 | 4.9 | 3.5 | 0.9 | 13.8 | 3.4 | 3.1 |
| Prulifloxacin-E2 | 2.0 | 7.8 | 12.6 | 3.3 | 1.3 | 2.1 | 2.1 | 6.0 | 5.5 | 7.5 | 2.2 | 4.5 |
| Ulifloxacin-E1 | 14.3 | 9.6 | 4.1 | 0.6 | 4.5 | 3.1 | 1.2 | 4.6 | 10.3 | 9.3 | 2.7 | 5.4 |
| Ulifloxacin-E2 | 7.2 | 14.8 | 8.6 | 13.3 | 2.8 | 3.7 | 7.0 | 2.1 | 10.8 | 10.2 | 6.6 | 6.6 |
| (±)-*cis*-Ketoconazole-E1 | 2.6 | 5.7 | 2.2 | 4.2 | 3.7 | 4.0 | 4.0 | 2.4 | 1.5 | 3.5 | 4.0 | 2.6 |
| (±)-*cis*-Ketoconazole-E2 | 2.9 | 2.0 | 6.8 | 0.3 | 2.2 | 2.6 | 2.7 | 1.4 | 1.9 | 3.9 | 1.7 | 2.0 |
| Flumequine-E1 | 3.8 | 2.2 | 4.1 | 0.7 | 5.2 | 1.8 | 4.7 | 2.3 | 0.4 | 3.4 | 2.6 | 2.5 |
| Flumequine-E2 | 8.6 | 11.3 | 6.8 | 1.3 | 2.8 | 7.7 | 4.6 | 5.1 | 0.9 | 8.9 | 3.9 | 3.5 |
| Nadifloxacin-E1 | 2.8 | 3.1 | 4.6 | 2.0 | 3.2 | 6.5 | 3.2 | 6.5 | 2.6 | 3.5 | 3.9 | 4.1 |
| Nadifloxacin-E2 | 4.3 | 11.3 | 10.2 | 6.3 | 3.1 | 5.0 | 5.9 | 6.4 | 8.1 | 8.6 | 4.8 | 6.8 |
| Besifloxacin | 3.0 | 2.1 | 8.5 | 13.2 | 7.9 | 2.5 | 12.4 | 3.2 | 2.7 | 4.5 | 7.9 | 6.1 |

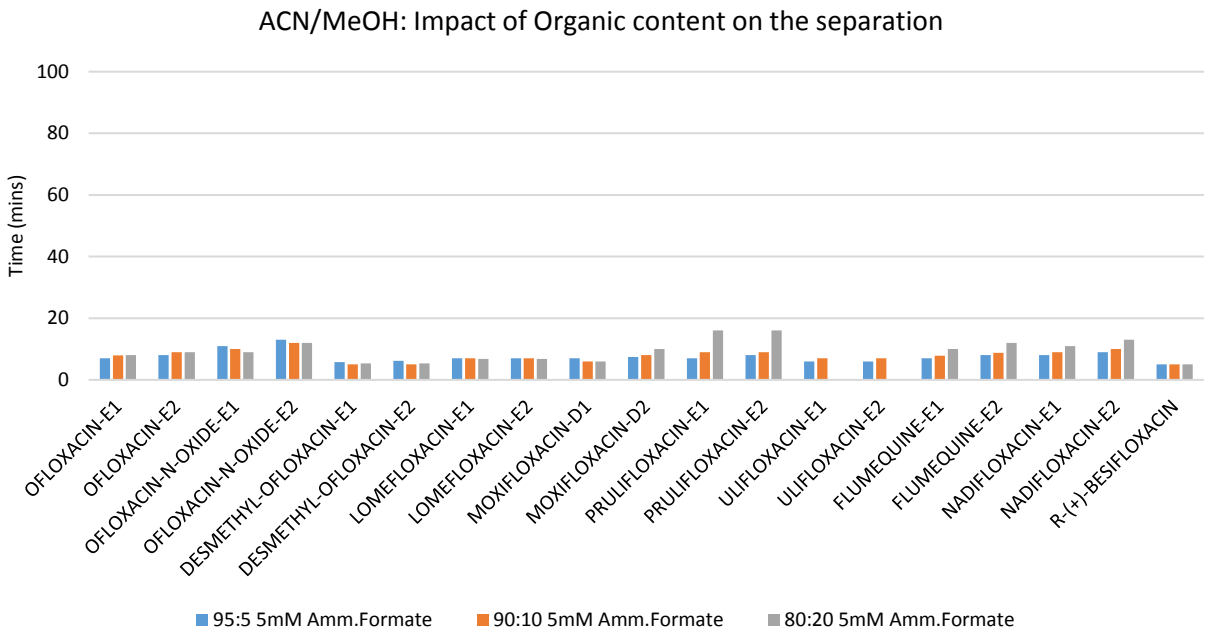
*-D indicates day

**- the following concentrations were used: 10, 100 and 1000 ng/L in the case of compounds that were not enantioseparated


ACN: Impact of Organic content on the separation
Time (mins)
100
80
60
40
20
0
OFLOXACIN-E1
OFLOXACIN-E2
OFLOXACIN-N-OXIDE-E1
OFLOXACIN-N-OXIDE-E2
DESMETHYL-OFLOXACIN-E1
DESMETHYL-OFLOXACIN-E2
LOMEFLOXACIN-E1
LOMEFLOXACIN-E2
MOXIFLOXACIN-D1
MOXIFLOXACIN-D2
PRULIFLOXACIN-E1
PRULIFLOXACIN-E2
ULIFLOXACIN-E1
ULIFLOXACIN-E2
FLUMEQUINE-E1
FLUMEQUINE-E2
NADIFLOXACIN-E1
NADIFLOXACIN-E2
R-(+)-BESIFLOXACIN
99:1 5mM Amm.Formate
95:5 5mM Amm.Formate
95:5 5mM Amm.Formate 0.1%FA pH 5.82
95:5 5mM Amm.Formate 0.2%FA pH 5.34
95:5 10mM Amm.Formate
95:5 1mM Amm.Formate
85:15 5mM Amm.Formate
75:25 5mM Amm.Formate
65:35 5mM Amm.Formate


EtOH: Impact of organic content on the separation
Time (mins)
100
80
60
40
20
0
OFLOXACIN-E1
OFLOXACIN-E2
OFLOXACIN-N-OXIDE-E1
OFLOXACIN-N-OXIDE-E2
DESMETHYL-OFLOXACIN-E1
DESMETHYL-OFLOXACIN-E2
LOMEFLOXACIN-E1
LOMEFLOXACIN-E2
MOXIFLOXACIN-D1
MOXIFLOXACIN-D2
PRULIFLOXACIN-E1
PRULIFLOXACIN-E2
ULIFLOXACIN-E1
ULIFLOXACIN-E2
FLUMEQUINE-E1
FLUMEQUINE-E2
NADIFLOXACIN-E1
NADIFLOXACIN-E2
R-(+)-BESIFLOXACIN


MeOH: Impact of organic content on the separation
Time (mins)
100
80
60
40
20
0
OFLOXACIN-E1
OFLOXACIN-E2
OFLOXACIN-N-OXIDE-E1
OFLOXACIN-N-OXIDE-E2
DESMETHYL-OFLOXACIN-E1
DESMETHYL-OFLOXACIN-E2
LOMEFLOXACIN-E1
LOMEFLOXACIN-E2
MOXIFLOXACIN-D1
MOXIFLOXACIN-D2
PRULIFLOXACIN-E1
PRULIFLOXACIN-E2
ULIFLOXACIN-E1
ULIFLOXACIN-E2
FLUMEQUINE-E1
FLUMEQUINE-E2
NADIFLOXACIN-E1
NADIFLOXACIN-E2
R-(+)-BESIFLOXACIN
99:1 10mM Amm.Formate 0.05%FA pH 5.50
99:1 10mM Amm.Formate
99:1 5mM Amm.Formate
95:5 10mM Amm.Formate 0.05%FA pH 5.38
95:5 5mM Amm.Formate 0.05%FA pH 5.14
95:5 5mM Amm.Formate
85:15 5mM Amm.Formate
75:25 5mM Amm.Formate
65:35 5mM Amm.Formate


ACN/MeOH: Impact of Organic content on the separation
Time (mins)
100
80
60
40
20
0
OFLOXACIN-E1
OFLOXACIN-E2
OFLOXACIN-N-OXIDE-E1
OFLOXACIN-N-OXIDE-E2
DESMETHYL-OFLOXACIN-E1
DESMETHYL-OFLOXACIN-E2
LOMEFLOXACIN-E1
LOMEFLOXACIN-E2
MOXIFLOXACIN-D1
MOXIFLOXACIN-D2
PRULIFLOXACIN-E1
PRULIFLOXACIN-E2
ULIFLOXACIN-E1
ULIFLOXACIN-E2
FLUMEQUINE-E1
FLUMEQUINE-E2
NADIFLOXACIN-E1
NADIFLOXACIN-E2
R-(+)-BESIFLOXACIN
95:5 5mM Amm.Formate
90:10 5mM Amm.Formate
80:20 5mM Amm.Formate

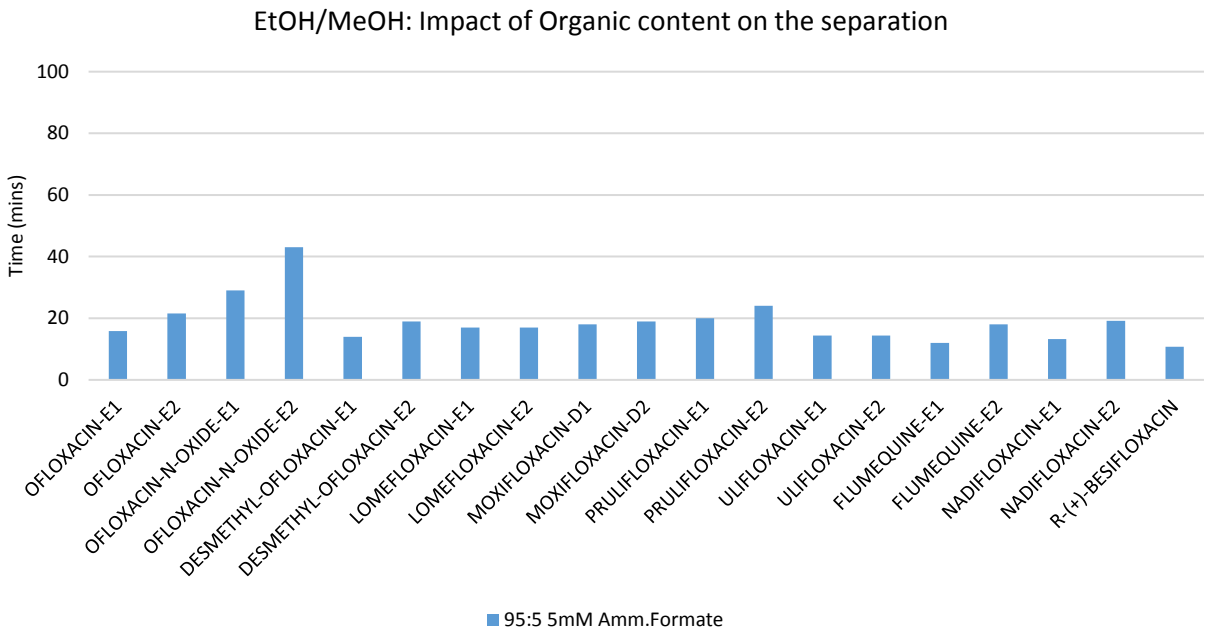
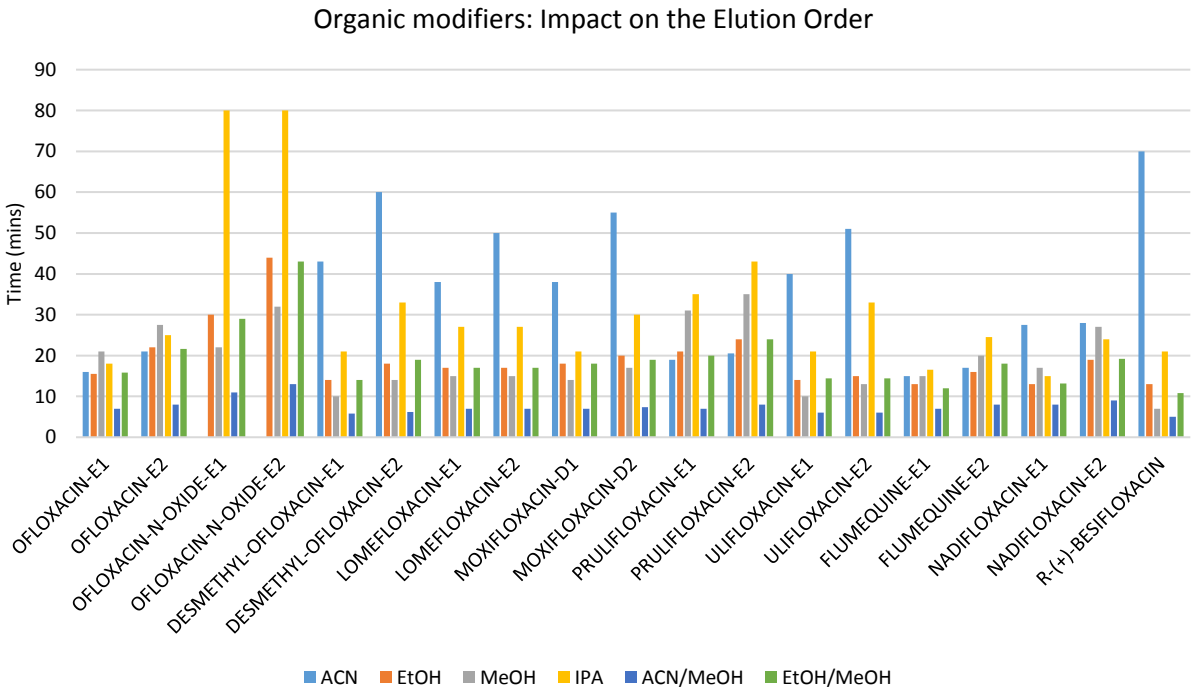



**Figure S1** CHIRALCEL® OZ-RH column – impact of the organic content on the separation of studied analytes (mobile phases in the legend are referred to the organic modifier mentioned in the title of the graphic).

(a)


Organic modifiers: Impact on the Elution Order
Time (mins)
90
80
70
60
50
40
30
20
10
0
OFLOXACIN-E1
OFLOXACIN-E2
OFLOXACIN-N-OXIDE-E1
OFLOXACIN-N-OXIDE-E2
DESMETHYL-OFLOXACIN-E1
DESMETHYL-OFLOXACIN-E2
LOMEFLOXACIN-E1
LOMEFLOXACIN-E2
MOXIFLOXACIN-D1
MOXIFLOXACIN-D2
PRULIFLOXACIN-E1
PRULIFLOXACIN-E2
ULIFLOXACIN-E1
ULIFLOXACIN-E2
FLUMEQUINE-E1
FLUMEQUINE-E2
NADIFLOXACIN-E1
NADIFLOXACIN-E2
R-(+)-BESIFLOXACIN
ACN
EtOH
MeOH
IPA
ACN/MeOH
EtOH/MeOH

(b)

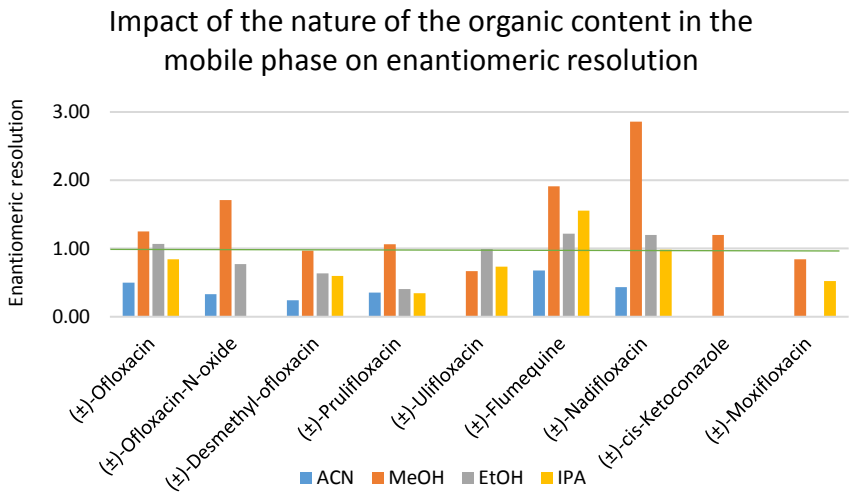
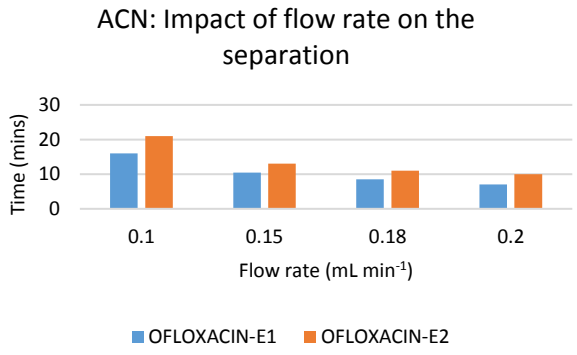
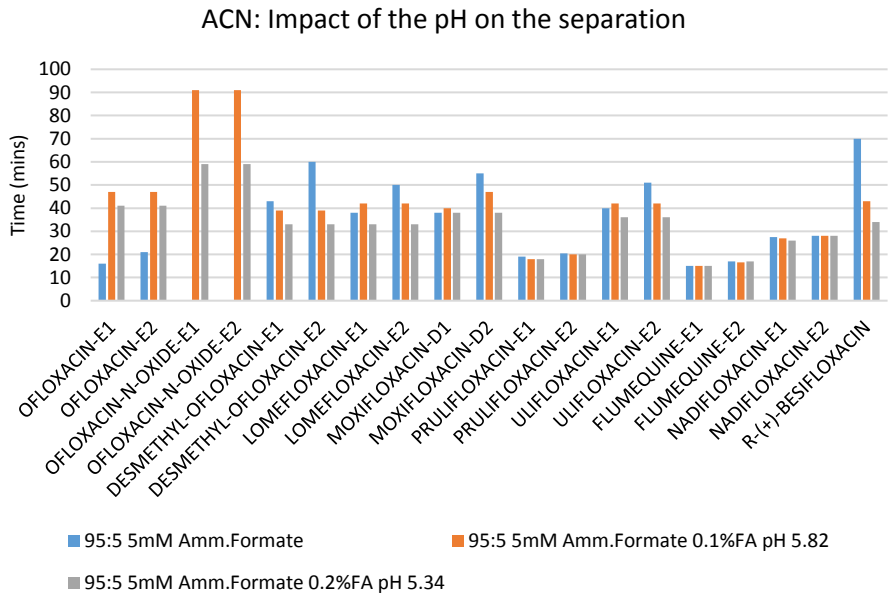



**Figure S2** CHIRALCEL® OZ-RH column – (a) impact of the organic modifiers used on the separation of studied analytes. The mobile phase composition was constituted by the organic solvent specified in the legend and by 5% of 5mM ammonium acetate as aqueous content; (b) Impact of the nature of the organic content in the mobile phase on enantiomeric resolution. The mobile phases considered were the best performing: 95:5 5mM ammonium formate for IPA and ACN, 99:1 5mM ammonium formate for EtOH, 99:1 10mM ammonium formate for MeOH.

(a)




(b)




(c)

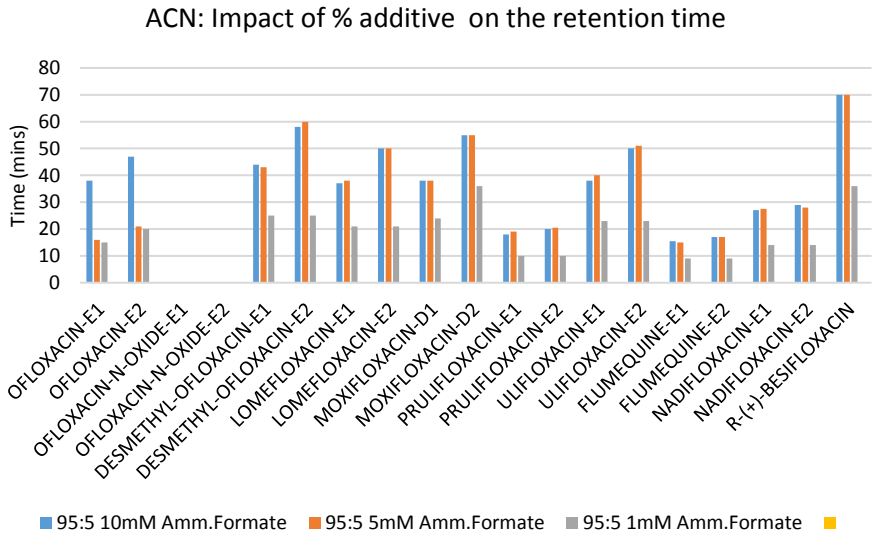



**Figure S3** CHIRALCEL® OZ-RH column – impact of (a) the flow rate, (b) pH and (c) percentage of the additive content on the separation of studied analytes. The mobile phase used for the first graphic was made of acetonitrile:water 95:5 5mM ammonium acetate.

# Appendix 5

The following supplementary data are contained in Appendix 5:

Table S1 Concentrations (Conc.), daily population-normalised mass loads (Loads) and EF values for chiral compounds for the European monitoring campaign across Europe (besifloxacin, *R*,*R*-moxifloxacin, prulifloxacin, nadifloxacin were not reported as all values were <MDL). M., T., W., T., F., S., S. were the initials used for indicating the days of the week.

Figure S1 Test "dry run on non-selective media" performed by using 100 µL (on the left) and 200 µL (on the right) of refrigerated wastewater Bristolian samples for day $6^{th}$ and $7^{th}$.

Figure S2 Growth of different colonies from an incubated Bristolian wastewater sample (on the left), result of the incubation for colony no. 7 and no.8 (on the centre), pointing of the single colony to be used a reference (on the right).

Figure S3 Result of the incubation for the colonies found from every day of the sampling campaign in Bristol.

Figure S4 Result of the incubation for the colonies found from every day of the sampling campaign in Oslo, Castellon, Lyngby, Milan, Utrecht and Zurich.

Table S2 Results on colony morphology assessment for the selected cities in the study.

Figure S5 qPCR-Melt curve.

Figure S6 qPCR-Standard curve.

**Table S1** Concentrations (Conc.), daily population-normalised mass loads (Loads) and EF values for chiral compounds for the European monitoring campaign across Europe (besifloxacin, *R,R*-moxifloxacin, prulifloxacin, nadifloxacin were not reported as all values were <MDL). M., T., W., T., F., S., S. were the initials used for indicating the days of the week.

| **CIPROFLOXACIN** | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 315.5 | 26.2 | 138.3 | 174.7 | 9.5 | 38.9 | 591.0 | 58.0 | 165.5 | 397.5 | 24.7 | 60.9 | 515.0 | 32.5 | 222.5 | 553.5 | 4.9 | 300.6 | 1867.5 | 142.1 | 387.3 |
| T | 352.5 | 14.8 | 153.4 | 192.0 | 7.2 | 44.3 | 565.0 | 62.2 | 160.6 | 365.0 | 33.9 | 54.2 | 1088.0 | 84.9 | 427.0 | 1166.0 | 173.9 | 448.5 | 1873.0 | 72.1 | 419.6 |
| W | 439.5 | 13.4 | 252.4 | 197.3 | 6.4 | 44.3 | 644.5 | 4.9 | 178.6 | 340.5 | 20.5 | 54.2 | 1100.0 | 65.1 | 421.4 | 1016.5 | 38.9 | 372.6 | 994.5 | 111.0 | 276.4 |
| T | 374.5 | 30.4 | 198.8 | 148.7 | 9.6 | 33.1 | 622.0 | 90.5 | 169.7 | 486.0 | 8.5 | 72.9 | 1005.0 | 56.6 | 394.7 | 1090.5 | 37.5 | 408.7 | 1969.5 | 229.8 | 535.8 |
| F | 325.5 | 19.1 | 155.5 | 124.3 | 5.9 | 35.4 | 601.5 | 95.5 | 164.5 | 304.0 | 2.8 | 50.2 | 460.5 | 6.4 | 181.3 | 923.0 | 69.3 | 337.5 | 2032.0 | 171.1 | 491.8 |
| S | 272.5 | 4.9 | 120.5 | 144.0 | 1.7 | 35.8 | 571.5 | 9.2 | 148.3 | 386.5 | 23.3 | 59.3 | 980.5 | 156.3 | 478.3 | 758.0 | 25.5 | 277.7 | 1805.0 | 55.2 | 382.6 |
| S | 341.5 | 17.7 | 147.3 | 143.7 | 4.5 | 31.3 | 595.0 | 41.0 | 153.8 | 378.0 | 32.5 | 59.1 | 544.5 | 10.6 | 322.7 | 756.0 | 35.4 | 290.5 | 1824.5 | 29.0 | 376.1 |
| AV | 345.9 | | 166.6 | 160.7 | | 37.6 | 598.6 | | 163.0 | 379.6 | | 58.7 | 813.4 | | 349.7 | 894.8 | | 348.0 | 1766.6 | | 409.9 |
| SD | 52.2 | | 44.7 | 27.5 | | 5.1 | 27.7 | | 10.0 | 56.5 | | 7.3 | 291.0 | | 111.8 | 216.8 | | 64.5 | 349.8 | | 84.5 |
| CV | 0.2 | | 0.3 | 0.2 | | 0.1 | 0.0 | | 0.1 | 0.1 | | 0.1 | 0.4 | | 0.3 | 0.2 | | 0.2 | 0.2 | | 0.2 |
| **DESETHYLENE-CIPROFLOXACIN** | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |

| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 26.0 | 36.8 | 11.4 | 60.7 | 1.5 | 13.5 | 66.5 | 2.1 | 18.6 | 78.5 | 6.4 | 12.0 | 50.5 | 2.1 | 21.8 | 59.5 | 0.7 | 32.3 | 68.5 | 3.5 | 14.2 |
| T | 51.0 | 0.0 | 22.2 | 57.5 | 0.7 | 13.3 | 71.5 | 0.7 | 20.3 | 76.0 | 1.4 | 11.3 | 52.0 | 0.0 | 20.4 | 0.0 | 0.0 | 0.0 | 66.0 | 1.4 | 14.8 |
| W | 91.5 | 4.9 | 52.6 | 53.3 | 2.1 | 12.0 | 69.0 | 1.4 | 19.1 | 65.5 | 4.9 | 10.4 | 26.0 | 36.8 | 10.0 | 0.0 | 0.0 | 0.0 | 66.0 | 1.4 | 18.3 |
| T | 40.5 | 57.3 | 21.5 | 53.7 | 1.5 | 12.0 | 73.5 | 7.8 | 20.0 | 75.0 | 0.0 | 11.3 | 51.0 | 0.0 | 20.0 | 0.0 | 0.0 | 0.0 | 69.5 | 6.4 | 18.9 |
| F | 30.0 | 42.4 | 14.3 | 53.7 | 1.2 | 15.3 | 68.0 | 0.0 | 18.6 | 71.5 | 2.1 | 11.8 | 25.0 | 35.4 | 9.8 | 26.0 | 36.8 | 9.5 | 69.0 | 1.4 | 16.7 |
| S | 50.0 | 1.4 | 22.1 | 53.0 | 1.7 | 13.2 | 70.0 | 0.0 | 18.2 | 77.0 | 7.1 | 11.8 | 51.0 | 1.4 | 24.9 | 0.0 | 0.0 | 0.0 | 67.0 | 2.8 | 14.2 |
| S | 53.0 | 0.0 | 22.9 | 53.3 | 1.5 | 11.6 | 71.5 | 2.1 | 18.5 | 72.0 | 4.2 | 11.3 | 50.5 | 0.7 | 29.9 | 55.0 | 2.8 | 21.1 | 65.0 | 1.4 | 13.4 |
| AV | 48.9 | | 23.9 | 55.0 | | 13.0 | 70.0 | | 19.1 | 73.6 | | 11.4 | 43.7 | | 19.6 | 20.1 | | 9.0 | 67.3 | | 15.8 |
| SD | 21.6 | | 13.4 | 2.9 | | 1.3 | 2.4 | | 0.8 | 4.4 | | 0.5 | 12.5 | | 7.4 | 27.1 | | 13.0 | 1.7 | | 2.2 |
| CV | 0.4 | | 0.6 | 0.1 | | 0.1 | 0.0 | | 0.0 | 0.1 | | 0.0 | 0.3 | | 0.4 | 1.4 | | 1.4 | 0.0 | | 0.1 |
| **NORFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 39.5 | 54.4 | 17.1 | 16.0 | 1.4 | 8.7 | 109.0 | 39.6 | 22.6 |
| T | 5.5 | 7.8 | 2.4 | 1.3 | 2.3 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 131.5 | 27.6 | 51.6 | 82.0 | 11.3 | 31.5 | 125.0 | 22.6 | 28.0 |
| W | 12.0 | 17.0 | 6.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 166.0 | 42.4 | 63.6 | 46.0 | 38.2 | 16.9 | 88.0 | 7.1 | 24.5 |
| T | 0.0 | 0.0 | 0.0 | 7.7 | 13.3 | 1.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 175.5 | 30.4 | 68.9 | 64.0 | 9.9 | 24.0 | 100.0 | 11.3 | 27.2 |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 0.0 | 0.0 | 0.0 | 9.3 | 11.4 | 2.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 20.5 | 10.6 | 8.1 | 55.5 | 13.4 | 20.3 | 113.0 | 4.2 | 27.3 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 105.5 | 40.3 | 51.5 | 34.5 | 13.4 | 12.6 | 117.5 | 21.9 | 24.9 |
| S | 11.0 | 15.6 | 4.7 | 5.3 | 9.2 | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 35.0 | 18.4 | 20.7 | 16.0 | 22.6 | 6.1 | 69.5 | 2.1 | 14.3 |
| AV | 4.1 | | 2.0 | 3.4 | | 0.8 | 0.0 | | 0.0 | 0.0 | | 0.0 | 96.2 | | 40.2 | 44.9 | | 17.2 | 103.1 | | 24.1 |
| SD | 5.5 | | 2.8 | 4.0 | | 1.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 64.8 | | 24.4 | 24.6 | | 8.9 | 19.1 | | 4.7 |
| CV | 1.3 | | 1.4 | 1.2 | | 1.3 | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! | 0.7 | | 0.6 | 0.5 | | 0.5 | 0.2 | | 0.2 |
| **NALIDIXIC ACID** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 4.5 | 0.7 | 2.0 | 5.3 | 0.6 | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.5 | 0.7 | 0.6 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.2 |
| T | 4.5 | 0.7 | 2.0 | 2.0 | 1.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.4 | 7.0 | 9.9 | 2.7 | 1.5 | 0.7 | 0.3 |
| W | 14.0 | 9.9 | 8.0 | 1.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 1.0 | 1.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.2 | 2.5 | 0.7 | 0.7 |
| T | 4.5 | 0.7 | 2.4 | 0.5 | 0.7 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.7 | 1.5 | 0.7 | 0.4 |
| F | 1.0 | 0.0 | 0.5 | 1.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 1.5 | 0.7 | 0.2 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.2 | 1.0 | 1.4 | 0.2 |
| S | 2.0 | 0.0 | 0.9 | 1.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 1.4 | 0.2 |
| S | 4.0 | 0.0 | 1.7 | 1.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.3 | 1.5 | 0.7 | 0.9 | 1.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 |
| AV | 4.9 | | 2.5 | 1.7 | | 0.4 | 0.0 | | 0.0 | 0.6 | | 0.1 | 0.6 | | 0.3 | 1.6 | | 0.6 | 1.2 | | 0.3 |
| SD | 4.2 | | 2.5 | 1.7 | | 0.4 | 0.0 | | 0.0 | 0.9 | | 0.1 | 0.7 | | 0.4 | 2.5 | | 1.0 | 0.8 | | 0.2 |
| CV | 0.9 | | 1.0 | 1.0 | | 0.9 | #DIV/0! | | #DIV/0! | 1.3 | | 1.3 | 1.3 | | 1.4 | 1.6 | | 1.6 | 0.6 | | 0.7 |
| **LOMEFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |

| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 1.5 | 2.1 | 0.7 | 2.7 | 0.6 | 0.6 | 3.5 | 0.7 | 1.0 | 3.5 | 0.7 | 0.5 | 1.5 | 2.1 | 0.6 | 3.0 | 0.0 | 1.6 | 1.5 | 2.1 | 0.3 |
| T | 3.0 | 1.4 | 1.3 | 2.5 | 0.7 | 0.6 | 1.5 | 2.1 | 0.4 | 1.5 | 2.1 | 0.2 | 0.0 | 0.0 | 0.0 | 14.0 | 14.1 | 5.4 | 1.0 | 1.4 | 0.2 |
| W | 11.0 | 5.7 | 6.3 | 2.3 | 0.6 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 1.1 | 6.5 | 0.7 | 2.4 | 3.5 | 2.1 | 1.0 |
| T | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.5 | 0.7 | 3.2 | 1.5 | 0.7 | 0.4 |
| F | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.1 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 1.8 | 1.5 | 2.1 | 0.4 |
| S | 0.5 | 0.7 | 0.2 | 1.3 | 0.6 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.5 | 0.7 | 1.6 | 4.0 | 0.0 | 0.8 |
| S | 2.0 | 2.8 | 0.9 | 2.0 | 1.0 | 0.4 | 1.5 | 2.1 | 0.4 | 1.0 | 1.4 | 0.2 | 0.0 | 0.0 | 0.0 | 5.5 | 2.1 | 2.1 | 2.0 | 0.0 | 0.4 |
| AV | 2.6 | | 1.3 | 2.3 | | 0.5 | 0.9 | | 0.3 | 0.9 | | 0.1 | 0.6 | | 0.3 | 6.7 | | 2.6 | 2.1 | | 0.5 |
| SD | 3.9 | | 2.2 | 0.5 | | 0.2 | 1.3 | | 0.4 | 1.3 | | 0.2 | 1.2 | | 0.5 | 3.6 | | 1.3 | 1.1 | | 0.3 |
| CV | 1.5 | | 1.7 | 0.2 | | 0.3 | 1.4 | | 1.5 | 1.4 | | 1.4 | 1.8 | | 1.8 | 0.5 | | 0.5 | 0.5 | | 0.6 |
| **MOXI-SULPHATE** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 80.5 | 10.6 | 12.3 | 124.5 | 21.9 | 53.8 | 0.0 | 0.0 | 0.0 | 505.5 | 17.7 | 104.8 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 117.0 | 56.6 | 33.3 | 11.0 | 15.6 | 1.6 | 78.0 | 14.1 | 30.6 | 84.0 | 7.1 | 32.3 | 484.5 | 9.2 | 108.5 |
| W | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 85.5 | 120.9 | 23.7 | 92.5 | 13.4 | 14.7 | 133.5 | 14.8 | 51.1 | 23.5 | 0.7 | 8.6 | 379.0 | 50.9 | 105.4 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 38.5 | 54.4 | 10.5 | 153.0 | 14.1 | 23.0 | 204.0 | 60.8 | 80.1 | 8.0 | 11.3 | 3.0 | 633.0 | 58.0 | 172.2 |
| F | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 96.0 | 14.1 | 15.8 | 138.5 | 13.4 | 54.5 | 19.0 | 4.2 | 6.9 | 794.5 | 55.9 | 192.3 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 46.0 | 65.1 | 11.9 | 108.0 | 17.0 | 16.6 | 105.0 | 8.5 | 51.2 | 0.0 | 0.0 | 0.0 | 1053.5 | 30.4 | 223.3 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 55.5 | 78.5 | 14.3 | 93.0 | 15.6 | 14.5 | 128.5 | 13.4 | 76.2 | 12.0 | 17.0 | 4.6 | 688.0 | 77.8 | 141.8 |
| AV | 0.0 | | 0.0 | 0.0 | | 0.0 | 48.9 | | 13.4 | 90.6 | | 14.1 | 130.3 | | 56.8 | 20.9 | | 7.9 | 648.3 | | 149.8 |
| SD | 0.0 | | 0.0 | 0.0 | | 0.0 | 42.7 | | 12.0 | 42.2 | | 6.4 | 38.6 | | 16.7 | 29.2 | | 11.2 | 226.2 | | 47.4 |
| CV | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! | 0.9 | | 0.9 | 0.5 | | 0.5 | 0.3 | | 0.3 | 1.4 | | 1.4 | 0.3 | | 0.3 |

| ***S*-(-)-OFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 42.0 | 0.0 | 18.4 | 50.3 | 1.2 | 11.2 | 29.0 | 2.8 | 8.1 | 29.5 | 0.7 | 4.5 | 153.0 | 5.7 | 66.1 | 1297.0 | 25.5 | 704.5 | 2592.5 | 147.8 | 537.6 |
| T | 38.0 | 1.4 | 16.5 | 38.3 | 6.0 | 8.8 | 22.5 | 2.1 | 6.4 | 28.5 | 0.7 | 4.2 | 360.5 | 13.4 | 141.5 | 2260.0 | 84.9 | 869.3 | 3011.5 | 88.4 | 674.6 |
| W | 41.5 | 13.4 | 23.8 | 42.7 | 2.1 | 9.6 | 25.0 | 0.0 | 6.9 | 20.0 | 2.8 | 3.2 | 337.5 | 14.8 | 129.3 | 2235.0 | 106.1 | 819.3 | 1849.0 | 76.4 | 514.0 |
| T | 36.5 | 0.7 | 19.4 | 31.7 | 2.1 | 7.1 | 23.0 | 2.8 | 6.3 | 24.0 | 1.4 | 3.6 | 302.5 | 9.2 | 118.8 | 2065.0 | 72.1 | 774.0 | 2878.5 | 26.2 | 783.2 |
| F | 28.0 | 4.2 | 13.4 | 31.3 | 1.2 | 8.9 | 19.5 | 2.1 | 5.3 | 15.0 | 0.0 | 2.5 | 139.5 | 6.4 | 54.9 | 1843.5 | 46.0 | 674.1 | 3315.5 | 74.2 | 802.4 |
| S | 31.0 | 1.4 | 13.7 | 46.3 | 2.5 | 11.5 | 19.5 | 2.1 | 5.1 | 22.5 | 0.7 | 3.5 | 331.5 | 3.5 | 161.7 | 1598.0 | 97.6 | 585.5 | 2812.5 | 70.0 | 596.2 |
| S | 35.5 | 2.1 | 15.3 | 45.0 | 1.7 | 9.8 | 22.5 | 0.7 | 5.8 | 25.0 | 2.8 | 3.9 | 165.0 | 5.7 | 97.8 | 1730.5 | 46.0 | 665.0 | 2743.5 | 62.9 | 565.5 |
| AV | 36.1 | | 17.2 | 40.8 | | 9.6 | 23.0 | | 6.3 | 23.5 | | 3.6 | 255.6 | | 110.0 | 1861.3 | | 727.4 | 2743.3 | | 639.1 |
| SD | 5.2 | | 3.7 | 7.3 | | 1.5 | 3.3 | | 1.0 | 5.0 | | 0.7 | 98.2 | | 39.2 | 352.4 | | 98.5 | 455.5 | | 116.8 |
| CV | 0.1 | | 0.2 | 0.2 | | 0.2 | 0.1 | | 0.2 | 0.2 | | 0.2 | 0.4 | | 0.4 | 0.2 | | 0.1 | 0.2 | | 0.2 |
| ***R*-(+)-OFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |

| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 6.0 | 1.4 | 2.6 | 13.3 | 0.6 | 3.0 | 0.0 | 0.0 | 0.0 | 6.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T | 9.5 | 3.5 | 4.1 | 12.3 | 1.2 | 2.8 | 0.0 | 0.0 | 0.0 | 4.0 | 1.4 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| W | 9.5 | 9.2 | 5.5 | 15.3 | 1.2 | 3.4 | 9.5 | 7.8 | 2.6 | 4.0 | 1.4 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T | 7.0 | 2.8 | 3.7 | 10.3 | 1.2 | 2.3 | 0.0 | 0.0 | 0.0 | 1.5 | 2.1 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| F | 7.0 | 0.0 | 3.3 | 10.3 | 1.2 | 2.9 | 0.0 | 0.0 | 0.0 | 3.5 | 0.7 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 8.5 | 2.1 | 3.8 | 14.3 | 3.2 | 3.6 | 4.0 | 0.0 | 1.0 | 5.5 | 2.1 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 6.0 | 1.4 | 2.6 | 13.7 | 2.9 | 3.0 | 4.5 | 0.7 | 1.2 | 4.0 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| AV | 7.6 | | 3.7 | 12.8 | | 3.0 | 2.6 | | 0.7 | 4.1 | | 0.6 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 |
| SD | 1.5 | | 1.0 | 1.9 | | 0.4 | 3.7 | | 1.0 | 1.5 | | 0.2 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 |
| CV | 0.2 | | 0.3 | 0.2 | | 0.1 | 1.4 | | 1.5 | 0.4 | | 0.4 | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! |
| **(±)-OFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 48.0 | 21.0 | 0.1 | 63.7 | 14.2 | 0.2 | 29.0 | 8.1 | 0.0 | 35.5 | 5.4 | 0.2 | 153.0 | 66.1 | 0.0 | 1297.0 | 704.5 | 0.0 | 2592.5 | 537.6 | 0.0 |
| T | 47.5 | 20.7 | 0.2 | 50.7 | 11.7 | 0.2 | 22.5 | 6.4 | 0.0 | 32.5 | 4.8 | 0.1 | 360.5 | 141.5 | 0.0 | 2260.0 | 869.3 | 0.0 | 3011.5 | 674.6 | 0.0 |
| W | 51.0 | 29.3 | 0.2 | 58.0 | 13.0 | 0.3 | 34.5 | 9.6 | 0.3 | 24.0 | 3.8 | 0.2 | 337.5 | 129.3 | 0.0 | 2235.0 | 819.3 | 0.0 | 1849.0 | 514.0 | 0.0 |
| T | 43.5 | 23.1 | 0.2 | 42.0 | 9.4 | 0.2 | 23.0 | 6.3 | 0.0 | 25.5 | 3.8 | 0.1 | 302.5 | 118.8 | 0.0 | 2065.0 | 774.0 | 0.0 | 2878.5 | 783.2 | 0.0 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F | 35.0 | 16.7 | 0.2 | 41.7 | 11.9 | 0.2 | 19.5 | 5.3 | 0.0 | 18.5 | 3.1 | 0.2 | 139.5 | 54.9 | 0.0 | 1843.5 | 674.1 | 0.0 | 3315.5 | 802.4 | 0.0 |
| S | 39.5 | 17.5 | 0.2 | 60.7 | 15.1 | 0.2 | 23.5 | 6.1 | 0.2 | 28.0 | 4.3 | 0.2 | 331.5 | 161.7 | 0.0 | 1598.0 | 585.5 | 0.0 | 2812.5 | 596.2 | 0.0 |
| S | 41.5 | 17.9 | 0.1 | 58.7 | 12.8 | 0.2 | 27.0 | 7.0 | 0.2 | 29.0 | 4.5 | 0.1 | 165.0 | 97.8 | 0.0 | 1730.5 | 665.0 | 0.0 | 2743.5 | 565.5 | 0.0 |
| AV | 43.7 | 20.9 | 0.2 | 53.6 | 12.6 | 0.2 | 25.6 | 7.0 | 0.1 | 27.6 | 4.3 | 0.1 | 255.6 | 110.0 | 0.0 | 1861.3 | 727.4 | 0.0 | 2743.3 | 639.1 | 0.0 |
| SD | 5.5 | 4.4 | 0.0 | 9.0 | 1.9 | 0.0 | 5.0 | 1.4 | 0.1 | 5.6 | 0.8 | 0.0 | 98.2 | 39.2 | 0.0 | 352.4 | 98.5 | 0.0 | 455.5 | 116.8 | 0.0 |
| CV | 0.1 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 0.2 | 0.2 | 1.3 | 0.2 | 0.2 | 0.3 | 0.4 | 0.4 | #DIV/0! | 0.2 | 0.1 | #DIV/0! | 0.2 | 0.2 | #DIV/0! |
| ***S*-(-)-OFLOXACIN-*N*-OXIDE** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 2.5 | 0.7 | 1.1 | 2.0 | 0.0 | 0.4 | 2.0 | 0.0 | 0.6 | 2.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 20.5 | 0.7 | 11.1 | 11.0 | 1.4 | 2.3 |
| T | 2.5 | 0.7 | 1.1 | 0.0 | 0.0 | 0.0 | 1.0 | 1.4 | 0.3 | 2.0 | 0.0 | 0.3 | 2.0 | 0.0 | 0.8 | 9.0 | 12.7 | 3.5 | 10.0 | 1.4 | 2.2 |
| W | 0.0 | 0.0 | 0.0 | 2.5 | 0.7 | 0.6 | 1.0 | 1.4 | 0.3 | 2.5 | 0.7 | 0.4 | 4.0 | 0.0 | 1.5 | 22.5 | 0.7 | 8.2 | 10.0 | 1.4 | 2.8 |
| T | 5.0 | 1.4 | 2.7 | 2.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.3 | 4.0 | 0.0 | 1.6 | 21.0 | 1.4 | 7.9 | 15.5 | 3.5 | 4.2 |
| F | 4.0 | 0.0 | 1.9 | 2.0 | 0.0 | 0.6 | 1.0 | 1.4 | 0.3 | 2.0 | 0.0 | 0.3 | 2.0 | 0.0 | 0.8 | 19.5 | 0.7 | 7.1 | 19.0 | 0.0 | 4.6 |
| S | 2.5 | 0.7 | 1.1 | 2.0 | 0.0 | 0.5 | 2.0 | 0.0 | 0.5 | 2.0 | 0.0 | 0.3 | 5.5 | 0.7 | 2.7 | 18.0 | 1.4 | 6.6 | 16.5 | 2.1 | 3.5 |
| S | 3.0 | 1.4 | 1.3 | 2.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.3 | 2.5 | 0.7 | 1.5 | 19.0 | 2.8 | 7.3 | 14.0 | 1.4 | 2.9 |
| AV | 2.8 | | 1.3 | 1.8 | | 0.4 | 1.0 | | 0.3 | 2.1 | | 0.3 | 2.9 | | 1.3 | 18.5 | | 7.4 | 13.7 | | 3.2 |
| SD | 1.6 | | 0.8 | 0.8 | | 0.2 | 0.8 | | 0.2 | 0.2 | | 0.0 | 1.8 | | 0.8 | 4.4 | | 2.3 | 3.5 | | 0.9 |
| CV | 0.6 | | 0.6 | 0.5 | | 0.5 | 0.8 | | 0.8 | 0.1 | | 0.1 | 0.6 | | 0.7 | 0.2 | | 0.3 | 0.3 | | 0.3 |
| ***R*-(+)-OFLOXACIN-*N*-OXIDE** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |

| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 2.0 | 0.0 | 0.9 | 2.0 | 0.0 | 0.4 | 1.0 | 1.4 | 0.3 | 1.0 | 1.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T | 2.0 | 0.0 | 0.9 | 0.7 | 1.2 | 0.2 | 0.0 | 0.0 | 0.0 | 1.0 | 1.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| W | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.4 | 1.0 | 1.4 | 0.3 | 1.0 | 1.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T | 2.5 | 0.7 | 1.3 | 2.0 | 0.0 | 0.4 | 1.0 | 1.4 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| F | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.6 | 2.0 | 0.0 | 0.5 | 1.0 | 1.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.5 | 2.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 2.0 | 0.0 | 0.9 | 2.0 | 0.0 | 0.4 | 1.0 | 1.4 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| AV | 1.2 | | 0.6 | 1.8 | | 0.4 | 1.1 | | 0.3 | 0.6 | | 0.1 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 |
| SD | 1.1 | | 0.6 | 0.5 | | 0.1 | 0.7 | | 0.2 | 0.5 | | 0.1 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 |
| CV | 0.9 | | 1.0 | 0.3 | | 0.3 | 0.6 | | 0.6 | 0.9 | | 0.9 | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! |
| **(±)-OFLOXACIN-*N*-OXIDE** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 4.5 | 2.0 | 0.4 | 4.0 | 0.9 | 0.5 | 3.0 | 0.8 | 0.3 | 3.0 | 0.5 | 0.3 | 0.0 | 0.0 | | 20.5 | 11.1 | 0.0 | 11.0 | 2.3 | 0.0 |
| T | 4.5 | 2.0 | 0.4 | 0.7 | 0.2 | 1.0 | 1.0 | 0.3 | 0.0 | 3.0 | 0.4 | 0.3 | 2.0 | 0.8 | 0.0 | 9.0 | 3.5 | 0.0 | 10.0 | 2.2 | 0.0 |
| W | 0.0 | 0.0 | | 4.5 | 1.0 | 0.4 | 2.0 | 0.6 | 0.5 | 3.5 | 0.6 | 0.3 | 4.0 | 1.5 | 0.0 | 22.5 | 8.2 | 0.0 | 10.0 | 2.8 | 0.0 |
| T | 7.5 | 4.0 | 0.3 | 4.0 | 0.9 | 0.5 | 1.0 | 0.3 | 1.0 | 2.0 | 0.3 | 0.0 | 4.0 | 1.6 | 0.0 | 21.0 | 7.9 | 0.0 | 15.5 | 4.2 | 0.0 |
| F | 4.0 | 1.9 | 0.0 | 4.0 | 1.1 | 0.5 | 3.0 | 0.8 | 0.7 | 3.0 | 0.5 | 0.3 | 2.0 | 0.8 | 0.0 | 19.5 | 7.1 | 0.0 | 19.0 | 4.6 | 0.0 |
| S | 2.5 | 1.1 | 0.0 | 4.0 | 1.0 | 0.5 | 4.0 | 1.0 | 0.5 | 2.0 | 0.3 | 0.0 | 5.5 | 2.7 | 0.0 | 18.0 | 6.6 | 0.0 | 16.5 | 3.5 | 0.0 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S | 5.0 | 2.2 | 0.4 | 4.0 | 0.9 | 0.5 | 1.0 | 0.3 | 1.0 | 2.0 | 0.3 | 0.0 | 2.5 | 1.5 | 0.0 | 19.0 | 7.3 | 0.0 | 14.0 | 2.9 | 0.0 |
| AV | 4.0 | 1.9 | 0.3 | 3.6 | 0.9 | 0.6 | 2.1 | 0.6 | 0.6 | 2.6 | 0.4 | 0.2 | 2.9 | 1.3 | 0.0 | 18.5 | 7.4 | 0.0 | 13.7 | 3.2 | 0.0 |
| SD | 2.3 | 1.2 | 0.2 | 1.3 | 0.3 | 0.2 | 1.2 | 0.3 | 0.4 | 0.6 | 0.1 | 0.2 | 1.8 | 0.8 | 0.0 | 4.4 | 2.3 | 0.0 | 3.5 | 0.9 | 0.0 |
| CV | 0.6 | 0.6 | 0.8 | 0.4 | 0.4 | 0.3 | 0.6 | 0.6 | 0.6 | 0.2 | 0.3 | 0.9 | 0.6 | 0.7 | #DIV/0! | 0.2 | 0.3 | #DIV/0! | 0.3 | 0.3 | #DIV/0! |
| ***S*-(-)-DESMETHYL-OFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 6.5 | 0.7 | 2.8 | 12.3 | 4.2 | 2.7 | 17.5 | 0.7 | 4.9 | 10.0 | 2.8 | 1.5 | 8.5 | 7.8 | 3.7 | 17.5 | 2.1 | 9.5 | 39.0 | 1.4 | 8.1 |
| T | 8.5 | 0.7 | 3.7 | 5.7 | 4.9 | 1.3 | 15.5 | 3.5 | 4.4 | 11.0 | 0.0 | 1.6 | 11.5 | 0.7 | 4.5 | 28.5 | 12.0 | 11.0 | 46.5 | 6.4 | 10.4 |
| W | 2.5 | 3.5 | 1.4 | 7.7 | 1.2 | 1.7 | 12.0 | 0.0 | 3.3 | 5.0 | 5.7 | 0.8 | 10.0 | 0.0 | 3.8 | 33.5 | 7.8 | 12.3 | 32.5 | 0.7 | 9.0 |
| T | 3.5 | 4.9 | 1.9 | 7.7 | 2.3 | 1.7 | 13.5 | 2.1 | 3.7 | 13.5 | 0.7 | 2.0 | 10.5 | 2.1 | 4.1 | 37.5 | 4.9 | 14.1 | 46.0 | 1.4 | 12.5 |
| F | 6.5 | 0.7 | 3.1 | 6.0 | 1.0 | 1.7 | 16.0 | 4.2 | 4.4 | 12.0 | 0.0 | 2.0 | 7.0 | 1.4 | 2.8 | 27.5 | 3.5 | 10.1 | 54.0 | 2.8 | 13.1 |
| S | 3.0 | 1.4 | 1.3 | 6.7 | 2.5 | 1.7 | 15.0 | 0.0 | 3.9 | 8.5 | 0.7 | 1.3 | 9.0 | 4.2 | 4.4 | 26.0 | 1.4 | 9.5 | 46.5 | 4.9 | 9.9 |
| S | 4.5 | 0.7 | 1.9 | 7.3 | 1.2 | 1.6 | 16.5 | 2.1 | 4.3 | 10.0 | 5.7 | 1.6 | 6.5 | 3.5 | 3.9 | 29.0 | 4.2 | 11.1 | 44.0 | 0.0 | 9.1 |
| AV | 5.0 | | 2.3 | 7.6 | | 1.8 | 15.1 | | 4.1 | 10.0 | | 1.5 | 9.0 | | 3.9 | 28.5 | | 11.1 | 44.1 | | 10.3 |
| SD | 2.2 | | 0.9 | 2.2 | | 0.5 | 1.9 | | 0.5 | 2.7 | | 0.4 | 1.8 | | 0.6 | 6.3 | | 1.6 | 6.8 | | 1.9 |
| CV | 0.4 | | 0.4 | 0.3 | | 0.3 | 0.1 | | 0.1 | 0.3 | | 0.3 | 0.2 | | 0.2 | 0.2 | | 0.1 | 0.2 | | 0.2 |
| ***R*-(+)-DESMETHYL-OFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 2.0 | 0.0 | 0.9 | 2.3 | 1.2 | 0.5 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T | 2.5 | 0.7 | 1.1 | 3.0 | 1.0 | 0.7 | 0.5 | 0.7 | 0.1 | 1.0 | 1.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| W | 12.0 | 17.0 | 6.9 | 3.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T | 2.5 | 3.5 | 1.3 | 3.7 | 0.6 | 0.8 | 0.0 | 0.0 | 0.0 | 2.5 | 0.7 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| F | 2.0 | 2.8 | 1.0 | 2.3 | 0.6 | 0.7 | 0.0 | 0.0 | 0.0 | 2.5 | 0.7 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 1.0 | 1.4 | 0.4 | 2.7 | 0.6 | 0.7 | 0.5 | 0.7 | 0.1 | 2.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 1.0 | 1.4 | 0.4 | 2.0 | 0.0 | 0.4 | 1.0 | 1.4 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| AV | 3.3 | | 1.7 | 2.7 | | 0.6 | 0.3 | | 0.1 | 1.5 | | 0.2 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | |
| SD | 3.9 | | 2.3 | 0.6 | | 0.1 | 0.4 | | 0.1 | 1.0 | | 0.2 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | |
| CV | 1.2 | | 1.3 | 0.2 | | 0.2 | 1.4 | | 1.4 | 0.7 | | 0.7 | #DIV/0! | | #DIV/0! | #DIV/0! | | #DIV/0! | #DIV/0! | |
| **(±)-DESMETHYL-OFLOXACIN** | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 8.5 | 3.7 | 0.2 | 14.7 | 3.3 | 0.2 | 17.5 | 4.9 | 0.0 | 12.0 | 1.8 | 0.2 | 8.5 | 3.7 | 0.0 | 17.5 | 9.5 | 0.0 | 39.0 | 8.1 | 0.0 |
| T | 11.0 | 4.8 | 0.2 | 8.7 | 2.0 | 0.3 | 16.0 | 4.5 | 0.0 | 12.0 | 1.8 | 0.1 | 11.5 | 4.5 | 0.0 | 28.5 | 11.0 | 0.0 | 46.5 | 10.4 | 0.0 |
| W | 14.5 | 8.3 | 0.8 | 10.7 | 2.4 | 0.3 | 12.0 | 3.3 | 0.0 | 5.5 | 0.9 | 0.1 | 10.0 | 3.8 | 0.0 | 33.5 | 12.3 | 0.0 | 32.5 | 9.0 | 0.0 |
| T | 6.0 | 3.2 | 0.4 | 11.3 | 2.5 | 0.3 | 13.5 | 3.7 | 0.0 | 16.0 | 2.4 | 0.2 | 10.5 | 4.1 | 0.0 | 37.5 | 14.1 | 0.0 | 46.0 | 12.5 | 0.0 |
| F | 8.5 | 4.1 | 0.2 | 8.3 | 2.4 | 0.3 | 16.0 | 4.4 | 0.0 | 14.5 | 2.4 | 0.2 | 7.0 | 2.8 | 0.0 | 27.5 | 10.1 | 0.0 | 54.0 | 13.1 | 0.0 |
| S | 4.0 | 1.8 | 0.3 | 9.3 | 2.3 | 0.3 | 15.5 | 4.0 | 0.0 | 10.5 | 1.6 | 0.2 | 9.0 | 4.4 | 0.0 | 26.0 | 9.5 | 0.0 | 46.5 | 9.9 | 0.0 |
| S | 5.5 | 2.4 | 0.2 | 9.3 | 2.0 | 0.2 | 17.5 | 4.5 | 0.1 | 10.0 | 1.6 | 0.0 | 6.5 | 3.9 | 0.0 | 29.0 | 11.1 | 0.0 | 44.0 | 9.1 | 0.0 |
| AV | 8.3 | 4.0 | 0.3 | 10.3 | 2.4 | 0.3 | 15.4 | 4.2 | 0.0 | 11.5 | 1.8 | 0.1 | 9.0 | 3.9 | 0.0 | 28.5 | 11.1 | 0.0 | 44.1 | 10.3 | 0.0 |
| SD | 3.6 | 2.1 | 0.2 | 2.2 | 0.4 | 0.1 | 2.0 | 0.5 | 0.0 | 3.4 | 0.5 | 0.1 | 1.8 | 0.6 | 0.0 | 6.3 | 1.6 | 0.0 | 6.8 | 1.9 | 0.0 |
| CV | 0.4 | 0.5 | 0.7 | 0.2 | 0.2 | 0.2 | 0.1 | 0.1 | 1.3 | 0.3 | 0.3 | 0.6 | 0.2 | 0.2 | #DIV/0! | 0.2 | 0.1 | #DIV/0! | 0.2 | 0.2 | #DIV/0! |

| ***S,S*-MOXIFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.0 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 | 10.0 | 1.4 | 4.3 | 6.0 | 0.0 | 3.3 | 74.5 | 13.4 | 15.4 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.0 | 1.4 | 2.3 | 0.0 | 0.0 | 0.0 | 14.5 | 0.7 | 5.7 | 6.5 | 9.2 | 2.5 | 84.5 | 2.1 | 18.9 |
| W | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.5 | 0.7 | 2.6 | 0.0 | 0.0 | 0.0 | 21.0 | 0.0 | 8.0 | 10.0 | 0.0 | 3.7 | 69.0 | 12.7 | 19.2 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.0 | 1.4 | 2.2 | 0.0 | 0.0 | 0.0 | 19.0 | 1.4 | 7.5 | 8.5 | 0.7 | 3.2 | 85.0 | 7.1 | 23.1 |
| F | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.0 | 1.4 | 1.6 | 0.0 | 0.0 | 0.0 | 7.5 | 0.7 | 3.0 | 7.0 | 0.0 | 2.6 | 121.5 | 6.4 | 29.4 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.5 | 2.1 | 1.9 | 0.0 | 0.0 | 0.0 | 17.0 | 1.4 | 8.3 | 9.0 | 2.8 | 3.3 | 127.0 | 9.9 | 26.9 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 1.3 | 0.0 | 0.0 | 0.0 | 10.5 | 0.7 | 6.2 | 7.5 | 0.7 | 2.9 | 87.5 | 2.1 | 18.0 |
| AV | 0.0 | | 0.0 | 0.0 | | 0.0 | 7.6 | | 2.1 | 0.0 | | 0.0 | 14.2 | | 6.1 | 7.8 | | 3.1 | 92.7 | | 21.6 |
| SD | 0.0 | | 0.0 | 0.0 | | 0.0 | 1.6 | | 0.5 | 0.0 | | 0.0 | 5.1 | | 2.0 | 1.4 | | 0.4 | 22.6 | | 5.1 |
| CV | - | | - | - | | - | 0.2 | | 0.2 | - | | - | 0.4 | | 0.3 | 0.2 | | 0.1 | 0.2 | | 0.2 |
| **(±)-MOXIFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 9.0 | 2.5 | 1.0 | 0.0 | 0.0 | - | 10.0 | 4.3 | 1.0 | 6.0 | 3.3 | 1.0 | 74.5 | 15.4 | 1.0 |
| T | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 8.0 | 2.3 | 1.0 | 0.0 | 0.0 | - | 14.5 | 5.7 | 1.0 | 6.5 | 2.5 | 1.0 | 84.5 | 18.9 | 1.0 |
| W | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 9.5 | 2.6 | 1.0 | 0.0 | 0.0 | - | 21.0 | 8.0 | 1.0 | 10.0 | 3.7 | 1.0 | 69.0 | 19.2 | 1.0 |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 8.0 | 2.2 | 1.0 | 0.0 | 0.0 | - | 19.0 | 7.5 | 1.0 | 8.5 | 3.2 | 1.0 | 85.0 | 23.1 | 1.0 |
| F | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 6.0 | 1.6 | 1.0 | 0.0 | 0.0 | - | 7.5 | 3.0 | 1.0 | 7.0 | 2.6 | 1.0 | 121.5 | 29.4 | 1.0 |
| S | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 7.5 | 1.9 | 1.0 | 0.0 | 0.0 | - | 17.0 | 8.3 | 1.0 | 9.0 | 3.3 | 1.0 | 127.0 | 26.9 | 1.0 |
| S | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 5.0 | 1.3 | 1.0 | 0.0 | 0.0 | - | 10.5 | 6.2 | 1.0 | 7.5 | 2.9 | 1.0 | 87.5 | 18.0 | 1.0 |
| AV | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 7.6 | 2.1 | 1.0 | 0.0 | 0.0 | - | 14.2 | 6.1 | 1.0 | 7.8 | 3.1 | 1.0 | 92.7 | 21.6 | 1.0 |
| SD | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 1.6 | 0.5 | 0.0 | 0.0 | 0.0 | - | 5.1 | 2.0 | 0.0 | 1.4 | 0.4 | 0.0 | 22.6 | 5.1 | 0.0 |
| CV | #DIV/0! | #DIV/0! | - | #DIV/0! | #DIV/0! | - | 0.2 | 0.2 | 0.0 | - | - | - | 0.4 | 0.3 | 0.0 | 0.2 | 0.1 | 0.0 | 0.2 | 0.2 | 0.0 |
| **ULIFLOXACIN-E1** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 49.5 | 9.2 | 26.9 | 0.0 | 0.0 | 0.0 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 21.5 | 3.5 | 8.3 | 0.0 | 0.0 | 0.0 |
| W | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 27.0 | 14.1 | 9.9 | 0.0 | 0.0 | 0.0 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 41.5 | 16.3 | 15.6 | 0.0 | 0.0 | 0.0 |
| F | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 29.5 | 6.4 | 10.8 | 0.0 | 0.0 | 0.0 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 43.0 | 1.4 | 15.8 | 0.0 | 0.0 | 0.0 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 31.0 | 4.2 | 11.9 | 0.0 | 0.0 | 0.0 |
| AV | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 34.7 | | 14.2 | 0.0 | | 0.0 |
| SD | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 10.1 | | 6.3 | 0.0 | | 0.0 |
| CV | - | | - | - | | - | - | | - | - | | - | - | | - | 0.3 | | 0.4 | - | | - |
| **ULIFLOXACIN-E2** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |

| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 25.0 | 7.1 | 13.6 | 0.0 | 0.0 | 0.0 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.0 | 15.6 | 4.2 | 0.0 | 0.0 | 0.0 |
| W | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 21.0 | 1.4 | 7.7 | 0.0 | 0.0 | 0.0 |
| T | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 22.5 | 2.1 | 8.4 | 0.0 | 0.0 | 0.0 |
| F | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.0 | 4.9 | 5.1 | 30.0 | 2.8 | 7.3 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 22.5 | 2.1 | 8.2 | 15.0 | 3.5 | 3.2 |
| S | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 26.0 | 8.5 | 10.0 | 0.0 | 3.5 | 0.0 |
| AV | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 20.3 | | 8.2 | 6.4 | | 1.5 |
| SD | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 5.6 | | 3.1 | 11.8 | | 2.8 |
| CV | - | | - | - | | - | - | | - | - | | - | - | | - | 0.3 | | 0.4 | 1.8 | | 1.9 |
| **(±)-ULIFLOXACIN** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 74.5 | 40.5 | 0.7 | 0.0 | 0.0 | - |
| T | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 32.5 | 12.5 | 0.7 | 0.0 | 0.0 | - |
| W | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 48.0 | 17.6 | 0.6 | 0.0 | 0.0 | - |
| T | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 64.0 | 24.0 | 0.6 | 0.0 | 0.0 | - |
| F | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 43.5 | 15.9 | 0.7 | 30.0 | 7.3 | - |
| S | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 65.5 | 24.0 | 0.7 | 15.0 | 3.2 | 0.0 |
| S | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 0.0 | 0.0 | | 57.0 | 21.9 | 0.5 | 0.0 | 0.0 | |

| | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AV | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 55.0 | 22.3 | 0.6 | 6.4 | 1.5 | - |
| SD | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 0.0 | 0.0 | - | 14.5 | 9.1 | 0.1 | 11.8 | 2.8 | - |
| CV | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.3 | 0.4 | 0.1 | 1.8 | 1.9 | - |
| **KETOCONAZOLE-D1** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 19.5 | 0.7 | 8.5 | 8.0 | 1.0 | 1.8 | 42.5 | 3.5 | 11.9 | 8.0 | 0.0 | 1.2 | 13.5 | 0.7 | 5.8 | 7.0 | 0.0 | 3.8 | 20.0 | 0.0 | 4.1 |
| T | 18.5 | 0.7 | 8.1 | 9.0 | 1.7 | 2.1 | 27.0 | 1.4 | 7.7 | 7.0 | 0.0 | 1.0 | 26.0 | 0.0 | 10.2 | 15.0 | 4.2 | 5.8 | 15.0 | 0.0 | 3.4 |
| W | 23.5 | 4.9 | 13.5 | 10.0 | 1.0 | 2.2 | 27.5 | 0.7 | 7.6 | 7.0 | 0.0 | 1.1 | 28.0 | 1.4 | 10.7 | 11.5 | 0.7 | 4.2 | 9.5 | 0.7 | 2.6 |
| T | 22.5 | 0.7 | 11.9 | 6.7 | 0.6 | 1.5 | 48.5 | 2.1 | 13.2 | 17.5 | 0.7 | 2.6 | 22.0 | 1.4 | 8.6 | 10.0 | 0.0 | 3.7 | 20.5 | 0.7 | 5.6 |
| F | 24.5 | 0.7 | 11.7 | 5.7 | 0.6 | 1.6 | 37.5 | 0.7 | 10.3 | 8.5 | 0.7 | 1.4 | 12.0 | 0.0 | 4.7 | 10.5 | 0.7 | 3.8 | 21.5 | 0.7 | 5.2 |
| S | 26.0 | 0.0 | 11.5 | 9.3 | 0.6 | 2.3 | 32.5 | 0.7 | 8.4 | 10.0 | 0.0 | 1.5 | 23.5 | 0.7 | 11.5 | 10.0 | 0.0 | 3.7 | 28.5 | 0.7 | 6.0 |
| S | 22.0 | 2.8 | 9.5 | 10.7 | 0.6 | 2.3 | 36.0 | 1.4 | 9.3 | 11.0 | 1.4 | 1.7 | 18.0 | 0.0 | 10.7 | 11.0 | 0.0 | 4.2 | 17.5 | 0.7 | 3.6 |
| AV | 22.4 | | 10.7 | 8.5 | | 2.0 | 35.9 | | 9.8 | 9.9 | | 1.5 | 20.4 | | 8.9 | 10.7 | | 4.2 | 18.9 | | 4.4 |
| SD | 2.7 | | 2.0 | 1.8 | | 0.3 | 7.8 | | 2.2 | 3.7 | | 0.5 | 6.1 | | 2.6 | 2.4 | | 0.7 | 5.9 | | 1.3 |
| CV | 0.1 | | 0.2 | 0.2 | | 0.2 | 0.2 | | 0.2 | 0.4 | | 0.4 | 0.3 | | 0.3 | 0.2 | | 0.2 | 0.3 | | 0.3 |
| **KETOCONAZOLE-D2** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 12.0 | 0.0 | 5.3 | 5.3 | 0.6 | 1.2 | 26.0 | 1.4 | 7.3 | 5.5 | 0.7 | 0.8 | 9.5 | 0.7 | 4.1 | 4.5 | 0.7 | 2.4 | 12.0 | 0.0 | 2.5 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| T | 11.5 | 0.7 | 5.0 | 5.3 | 0.6 | 1.2 | 15.0 | 0.0 | 4.3 | 5.0 | 0.0 | 0.7 | 18.0 | 0.0 | 7.1 | 11.0 | 2.8 | 4.2 | 9.5 | 0.7 | 2.1 |
| W | 18.0 | 2.8 | 10.3 | 6.0 | 0.0 | 1.3 | 16.0 | 0.0 | 4.4 | 4.5 | 0.7 | 0.7 | 20.0 | 0.0 | 7.7 | 7.5 | 0.7 | 2.7 | 6.5 | 0.7 | 1.8 |
| T | 16.5 | 0.7 | 8.8 | 4.0 | 0.0 | 0.9 | 33.0 | 1.4 | 9.0 | 10.5 | 0.7 | 1.6 | 15.5 | 0.7 | 6.1 | 7.0 | 0.0 | 2.6 | 13.0 | 1.4 | 3.5 |
| F | 16.5 | 0.7 | 7.9 | 4.0 | 0.0 | 1.1 | 20.0 | 0.0 | 5.5 | 6.0 | 0.0 | 1.0 | 7.0 | 1.4 | 2.8 | 8.0 | 0.0 | 2.9 | 12.0 | 1.4 | 2.9 |
| S | 17.5 | 2.1 | 7.7 | 6.0 | 0.0 | 1.5 | 16.5 | 0.7 | 4.3 | 6.5 | 0.7 | 1.0 | 16.0 | 0.0 | 7.8 | 7.0 | 0.0 | 2.6 | 16.0 | 0.0 | 3.4 |
| S | 14.0 | 1.4 | 6.0 | 6.0 | 0.0 | 1.3 | 20.0 | 1.4 | 5.2 | 7.0 | 0.0 | 1.1 | 12.0 | 0.0 | 7.1 | 7.5 | 0.7 | 2.9 | 11.5 | 0.7 | 2.4 |
| AV | 15.1 | | 7.3 | 5.2 | | 1.2 | 20.9 | | 5.7 | 6.4 | | 1.0 | 14.0 | | 6.1 | 7.5 | | 2.9 | 11.5 | | 2.7 |
| SD | 2.6 | | 2.0 | 0.9 | | 0.2 | 6.5 | | 1.8 | 2.0 | | 0.3 | 4.7 | | 1.9 | 1.9 | | 0.6 | 2.9 | | 0.6 |
| CV | 0.2 | | 0.3 | 0.2 | | 0.2 | 0.3 | | 0.3 | 0.3 | | 0.3 | 0.3 | | 0.3 | 0.3 | | 0.2 | 0.3 | | 0.2 |
| **(±)-KETOCONAZOLE** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 31.5 | 13.8 | 0.6 | 13.3 | 3.0 | 0.6 | 68.5 | 19.2 | 0.6 | 13.5 | 2.1 | 0.6 | 23.0 | 9.9 | 0.6 | 11.5 | 6.2 | 0.6 | 32.0 | 6.6 | 0.6 |
| T | 30.0 | 13.1 | 0.6 | 14.3 | 3.3 | 0.6 | 42.0 | 11.9 | 0.6 | 12.0 | 1.8 | 0.6 | 44.0 | 17.3 | 0.6 | 26.0 | 10.0 | 0.6 | 24.5 | 5.5 | 0.6 |
| W | 41.5 | 23.8 | 0.6 | 16.0 | 3.6 | 0.6 | 43.5 | 12.1 | 0.6 | 11.5 | 1.8 | 0.6 | 48.0 | 18.4 | 0.6 | 19.0 | 7.0 | 0.6 | 16.0 | 4.4 | 0.6 |
| T | 39.0 | 20.7 | 0.6 | 10.7 | 2.4 | 0.6 | 81.5 | 22.2 | 0.6 | 28.0 | 4.2 | 0.6 | 37.5 | 14.7 | 0.6 | 17.0 | 6.4 | 0.6 | 33.5 | 9.1 | 0.6 |
| F | 41.0 | 19.6 | 0.6 | 9.7 | 2.8 | 0.6 | 57.5 | 15.7 | 0.7 | 14.5 | 2.4 | 0.6 | 19.0 | 7.5 | 0.6 | 18.5 | 6.8 | 0.6 | 33.5 | 8.1 | 0.6 |
| S | 43.5 | 19.2 | 0.6 | 15.3 | 3.8 | 0.6 | 49.0 | 12.7 | 0.7 | 16.5 | 2.5 | 0.6 | 39.5 | 19.3 | 0.6 | 17.0 | 6.2 | 0.6 | 44.5 | 9.4 | 0.6 |
| S | 36.0 | 15.5 | 0.6 | 16.7 | 3.6 | 0.6 | 56.0 | 14.5 | 0.6 | 18.0 | 2.8 | 0.6 | 30.0 | 17.8 | 0.6 | 18.5 | 7.1 | 0.6 | 29.0 | 6.0 | 0.6 |
| AV | 37.5 | 18.0 | 0.6 | 13.7 | 3.2 | 0.6 | 56.9 | 15.5 | 0.6 | 16.3 | 2.5 | 0.6 | 34.4 | 15.0 | 0.6 | 18.2 | 7.1 | 0.6 | 30.4 | 7.0 | 0.6 |
| SD | 5.2 | 3.9 | 0.0 | 2.7 | 0.5 | 0.0 | 14.2 | 3.9 | 0.0 | 5.7 | 0.8 | 0.0 | 10.8 | 4.6 | 0.0 | 4.3 | 1.3 | 0.0 | 8.8 | 1.9 | 0.0 |
| CV | 0.1 | 0.2 | 0.0 | 0.2 | 0.2 | 0.0 | 0.2 | 0.3 | 0.0 | 0.3 | 0.3 | 0.0 | 0.3 | 0.3 | 0.0 | 0.2 | 0.2 | 0.0 | 0.3 | 0.3 | 0.0 |
| **FLUMEQUINE-E1** | | | | | | | | | | | | | | | | | | | | | |

| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 1.0 | 0.0 | 0.4 | 0.7 | 0.6 | 0.1 | 1.5 | 0.7 | 0.4 | 1.5 | 0.7 | 0.2 | 1.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 1.5 | 0.7 | 0.3 |
| T | 1.0 | 0.0 | 0.4 | 1.7 | 0.6 | 0.4 | 1.0 | 0.0 | 0.3 | 2.0 | 0.0 | 0.3 | 3.5 | 0.7 | 1.4 | 3.0 | 2.8 | 1.2 | 2.0 | 0.0 | 0.4 |
| W | 2.5 | 2.1 | 1.4 | 1.0 | 0.0 | 0.2 | 1.0 | 0.0 | 0.3 | 1.5 | 0.7 | 0.2 | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.4 | 1.5 | 0.7 | 0.4 |
| T | 1.0 | 0.0 | 0.5 | 0.3 | 0.6 | 0.1 | 1.0 | 0.0 | 0.3 | 3.5 | 0.7 | 0.5 | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.4 | 3.5 | 0.7 | 1.0 |
| F | 1.0 | 0.0 | 0.5 | 1.0 | 0.0 | 0.3 | 1.0 | 0.0 | 0.3 | 1.5 | 0.7 | 0.2 | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.2 |
| S | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.2 | 2.0 | 0.0 | 0.5 | 1.5 | 0.7 | 0.2 | 3.0 | 0.0 | 1.5 | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.2 |
| S | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.2 | 1.0 | 0.0 | 0.3 | 1.5 | 0.7 | 0.2 | 1.0 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 1.5 | 0.7 | 0.3 |
| AV | 1.2 | | 0.6 | 1.0 | | 0.2 | 1.2 | | 0.3 | 1.9 | | 0.3 | 1.6 | | 0.7 | 1.0 | | 0.4 | 1.7 | | 0.4 |
| SD | 0.6 | | 0.4 | 0.4 | | 0.1 | 0.4 | | 0.1 | 0.7 | | 0.1 | 1.1 | | 0.5 | 1.0 | | 0.4 | 0.9 | | 0.3 |
| CV | 0.5 | | 0.6 | 0.4 | | 0.4 | 0.3 | | 0.3 | 0.4 | | 0.4 | 0.7 | | 0.7 | 1.0 | | 1.0 | 0.5 | | 0.6 |
| **FLUMEQUINE-E2** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) | CONC. (ng/L) | SD | LOADS (mg/1000 people/day) |
| M | 0.0 | 0.0 | 0.0 | 0.3 | 0.6 | 0.1 | 0.5 | 0.7 | 0.1 | 1.0 | 0.0 | 0.2 | 0.5 | 0.7 | 0.2 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.1 |
| T | 0.0 | 0.0 | 0.0 | 1.7 | 0.6 | 0.4 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.1 | 3.0 | 0.0 | 1.2 | 4.5 | 4.9 | 1.7 | 1.0 | 0.0 | 0.2 |
| W | 3.0 | 2.8 | 1.7 | 0.3 | 0.6 | 0.1 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.2 | 0.5 | 0.7 | 0.2 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.1 |
| T | 1.0 | 0.0 | 0.5 | 0.3 | 0.6 | 0.1 | 0.0 | 0.0 | 0.0 | 3.0 | 0.0 | 0.5 | 1.0 | 0.0 | 0.4 | 0.5 | 0.7 | 0.2 | 2.5 | 0.7 | 0.7 |
| F | 0.5 | 0.7 | 0.2 | 0.3 | 0.6 | 0.1 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.2 | 0.5 | 0.7 | 0.2 | 0.5 | 0.7 | 0.2 | 0.5 | 0.7 | 0.1 |
| S | 1.0 | 0.0 | 0.4 | 1.0 | 0.0 | 0.2 | 1.0 | 0.0 | 0.3 | 1.0 | 0.0 | 0.2 | 2.5 | 0.7 | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.2 | 0.5 | 0.7 | 0.3 | 0.0 | 0.0 | 0.0 | 0.5 | 0.7 | 0.1 |
| AV | 0.8 | | 0.4 | 0.7 | | 0.2 | 0.2 | | 0.1 | 1.3 | | 0.2 | 1.2 | | 0.5 | 0.8 | | 0.3 | 0.8 | | 0.2 |
| SD | 1.1 | | 0.6 | 0.5 | | 0.1 | 0.4 | | 0.1 | 0.8 | | 0.1 | 1.1 | | 0.5 | 1.7 | | 0.6 | 0.8 | | 0.2 |
| CV | 1.4 | | 1.5 | 0.7 | | 0.7 | 1.8 | | 1.8 | 0.6 | | 0.6 | 0.9 | | 0.9 | 2.1 | | 2.1 | 1.0 | | 1.1 |
| **(±)-FLUMEQUINE** | | | | | | | | | | | | | | | | | | | | | |
| | **Norway** | | | **UK** | | | **Denmark** | | | **Netherlands** | | | **Switzerland** | | | **Italy** | | | **Spain** | | |
| | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF | CONC. (ng/L) | LOADS (mg/1000 people/day) | EF |
| M | 1.0 | 0.4 | 1.0 | 1.0 | 0.2 | 0.7 | 2.0 | 0.6 | 0.8 | 2.5 | 0.4 | 0.6 | 1.5 | 0.6 | 0.7 | 0.0 | 0.0 | | 2.0 | 0.0 | 0.8 |
| T | 1.0 | 0.4 | 1.0 | 3.3 | 0.8 | 0.5 | 1.0 | 0.3 | 1.0 | 3.0 | 0.4 | 0.7 | 6.5 | 2.6 | 0.5 | 7.5 | 2.9 | 0.4 | 3.0 | 0.0 | 0.7 |
| W | 5.5 | 3.2 | 0.5 | 1.3 | 0.3 | 0.8 | 1.0 | 0.3 | 1.0 | 2.5 | 0.4 | 0.6 | 1.5 | 0.6 | 0.7 | 1.0 | 0.4 | 1.0 | 2.0 | 0.0 | 0.8 |
| T | 2.0 | 1.1 | 0.5 | 0.7 | 0.1 | 0.5 | 1.0 | 0.3 | 1.0 | 6.5 | 1.0 | 0.5 | 2.0 | 0.8 | 0.5 | 1.5 | 0.6 | 0.7 | 6.0 | 0.0 | 0.6 |
| F | 1.5 | 0.7 | 0.7 | 1.3 | 0.4 | 0.8 | 1.0 | 0.3 | 1.0 | 2.5 | 0.4 | 0.6 | 1.5 | 0.6 | 0.7 | 1.5 | 0.5 | 0.7 | 1.5 | 0.0 | 0.7 |
| S | 2.0 | 0.9 | 0.5 | 2.0 | 0.5 | 0.5 | 3.0 | 0.8 | 0.7 | 2.5 | 0.4 | 0.6 | 5.5 | 2.7 | 0.5 | 1.0 | 0.4 | 1.0 | 1.0 | 0.0 | 1.0 |
| S | 1.0 | 0.4 | 1.0 | 2.0 | 0.4 | 0.5 | 1.0 | 0.3 | 1.0 | 2.5 | 0.4 | 0.6 | 1.5 | 0.9 | 0.7 | 0.0 | 0.0 | | 2.0 | 0.0 | 0.8 |
| AV | 2.0 | 1.0 | 0.7 | 1.7 | 0.4 | 0.6 | 1.4 | 0.4 | 0.9 | 3.1 | 0.5 | 0.6 | 2.9 | 1.2 | 0.6 | 1.8 | 0.7 | 0.7 | 2.5 | 0.0 | 0.7 |
| SD | 1.6 | 1.0 | 0.3 | 0.9 | 0.2 | 0.1 | 0.8 | 0.2 | 0.1 | 1.5 | 0.2 | 0.0 | 2.2 | 0.9 | 0.1 | 2.6 | 1.0 | 0.3 | 1.7 | 0.0 | 0.1 |
| CV | 0.8 | 1.0 | 0.4 | 0.5 | 0.5 | 0.2 | 0.6 | 0.5 | 0.2 | 0.5 | 0.4 | 0.1 | 0.8 | 0.8 | 0.1 | 1.5 | 1.5 | 0.3 | 0.7 | - | 0.2 |

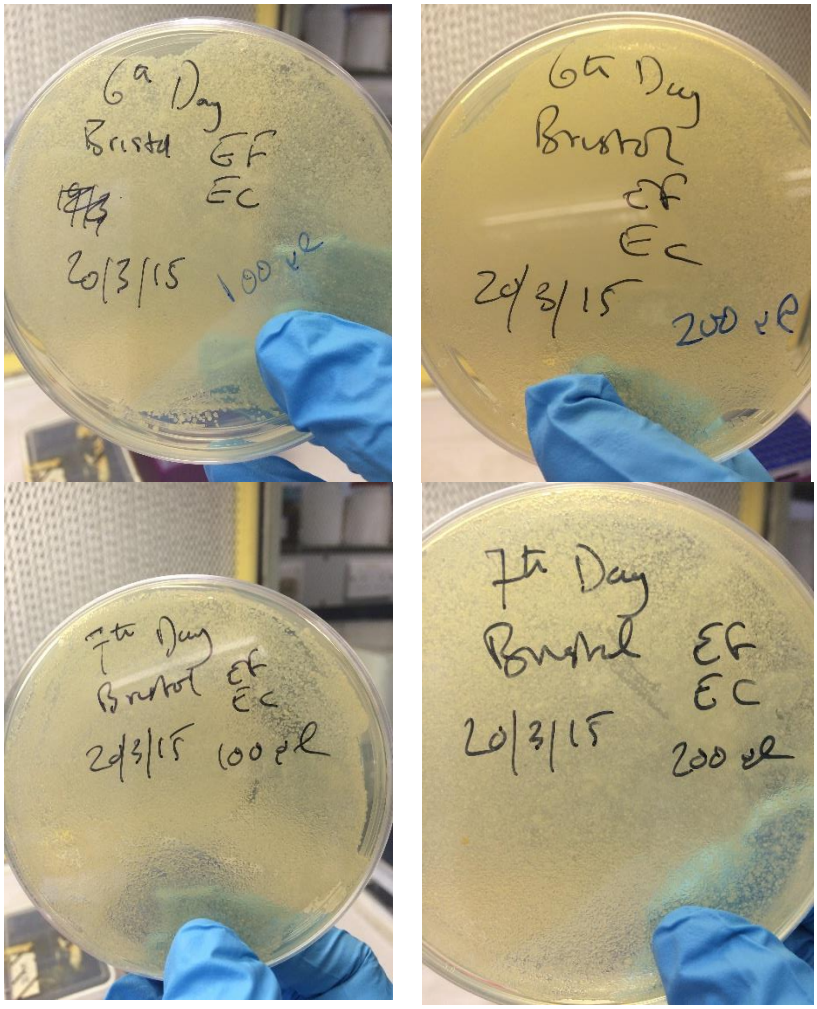



**Figure S1** Test "dry run on non-selective media" performed by using 100 µL (on the left) and 200 µL (on the right) of refrigerated wastewater Bristolian samples for day 6$^{th}$ and 7$^{th}$.

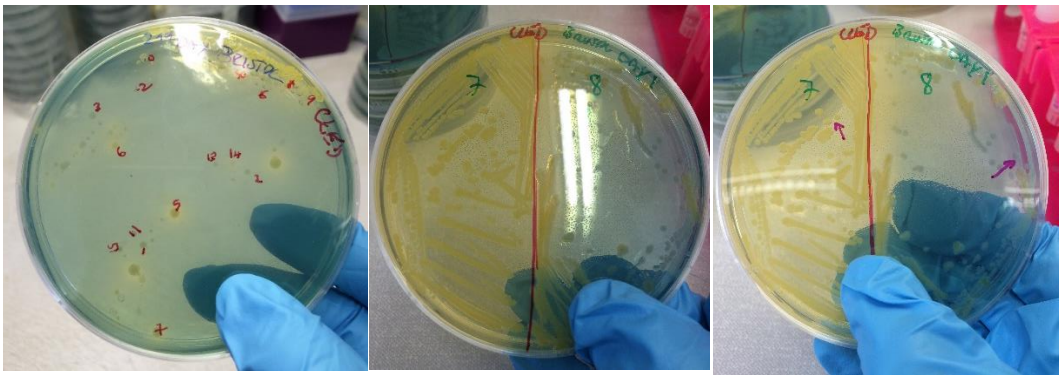
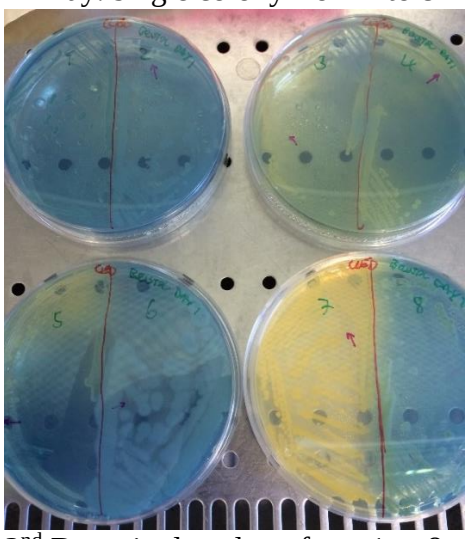


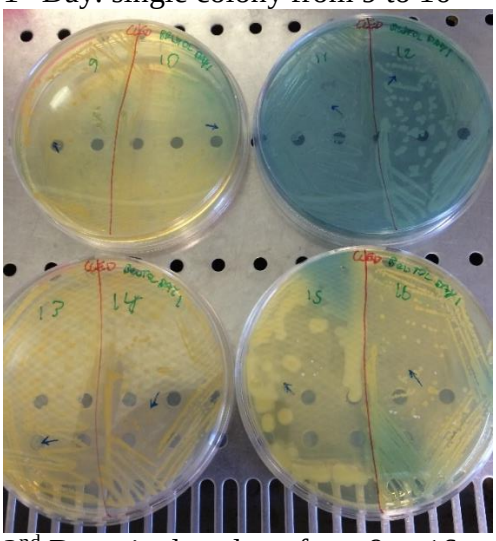
**Figure S2** Growth of different colonies from an incubated Bristolian wastewater sample (on the left), result of the incubation for colony no. 7 and no.8 (on the centre), pointing of the single colony to be used a reference (on the right).

BRISTOL

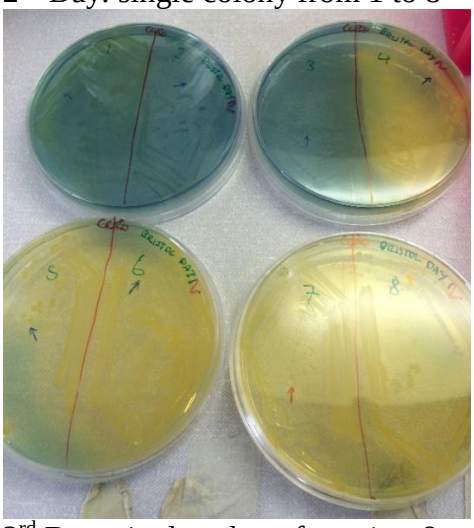
1st Day: single colony from 1 to 8



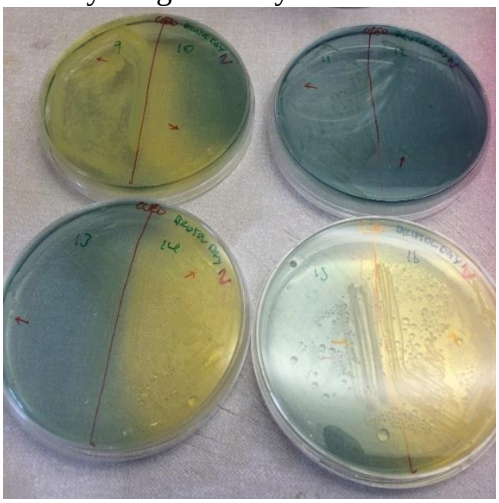
1st Day: single colony from 9 to 16



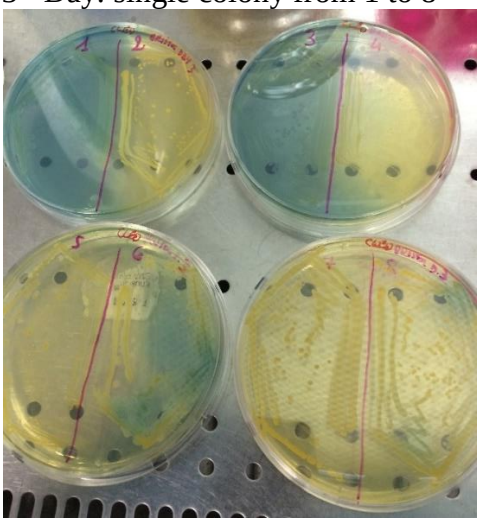
2nd Day: single colony from 1 to 8



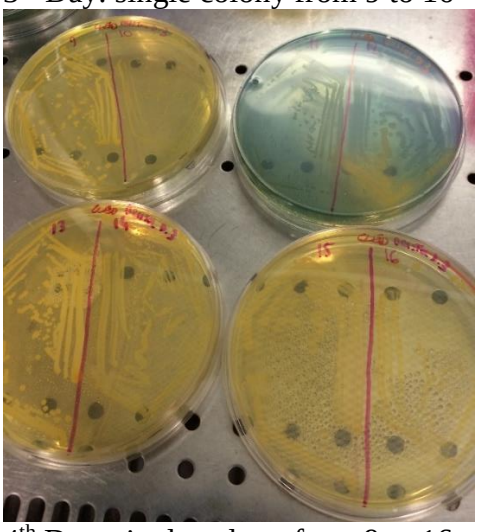
2nd Day: single colony from 9 to 16



3rd Day: single colony from 1 to 8

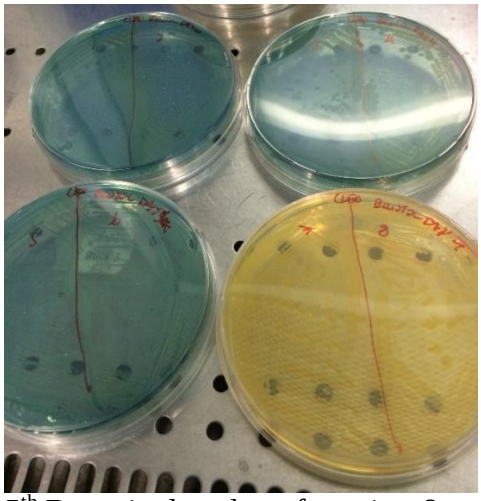


3rd Day: single colony from 9 to 16

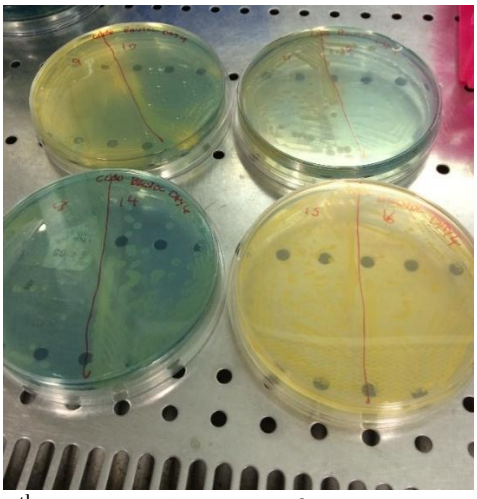


4th Day: single colony from 1 to 8

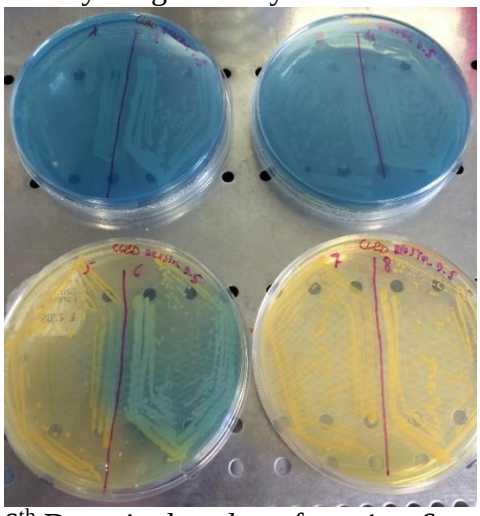
4th Day: single colony from 9 to 16



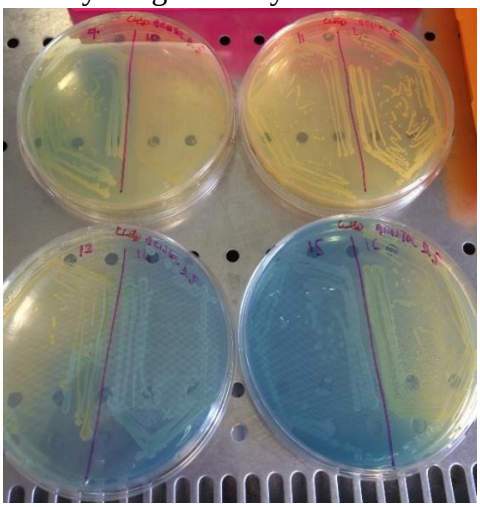
5th Day: single colony from 1 to 8



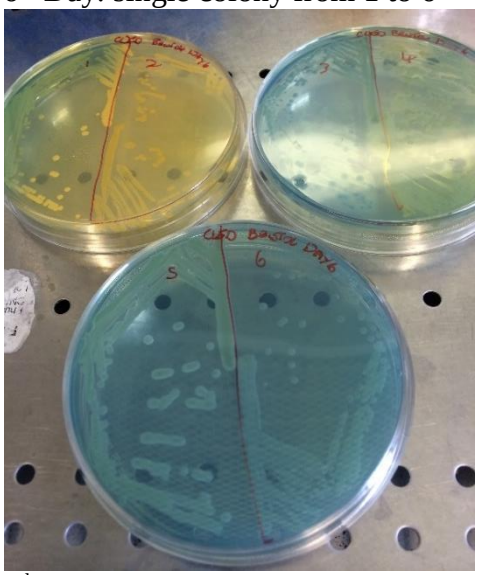
5th Day: single colony from 9 to 16



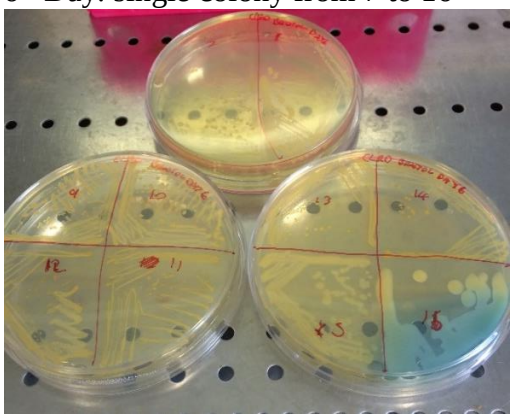
6th Day: single colony from 1 to 6



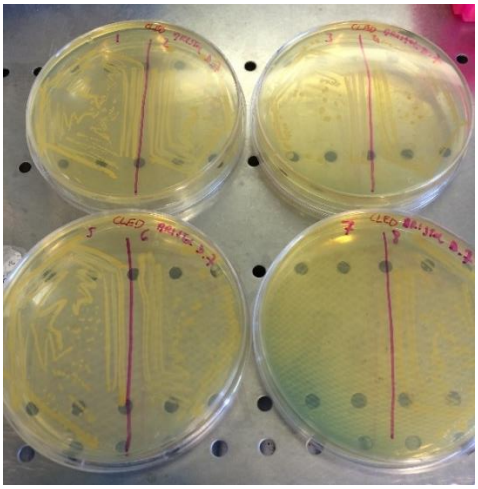
6th Day: single colony from 7 to 16



7th Day: single colony from 1 to 8



7th Day: single colony from 9 to 16



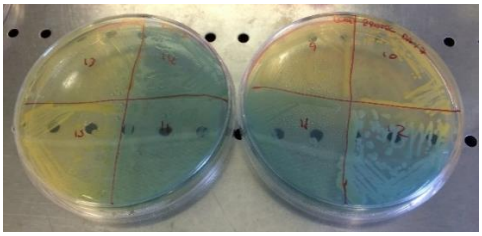
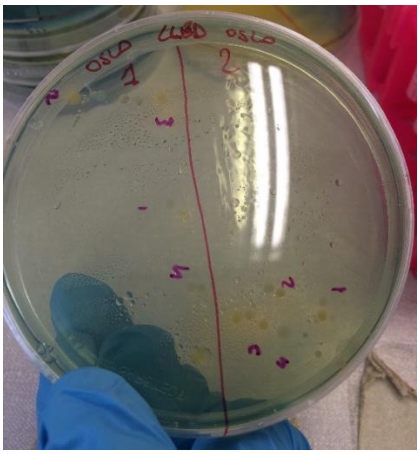


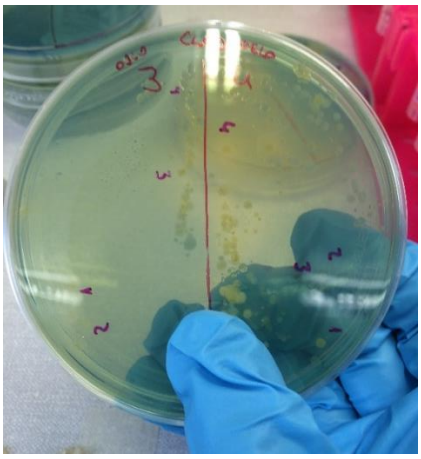
**Figure S3** Result of the incubation for the colonies found from every day of the sampling campaign in Bristol.

OSLO

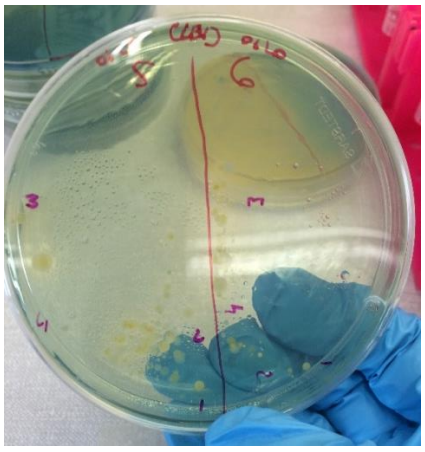
1st and 2nd Day: single colony from 1 to 4



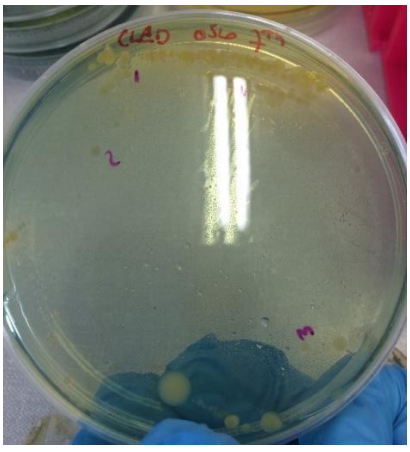
3rd and 4th Day: single colony from 1 to 4



5th and 6th Day: single colony from 1 to 4



7th Day: single colony from 1 to 4

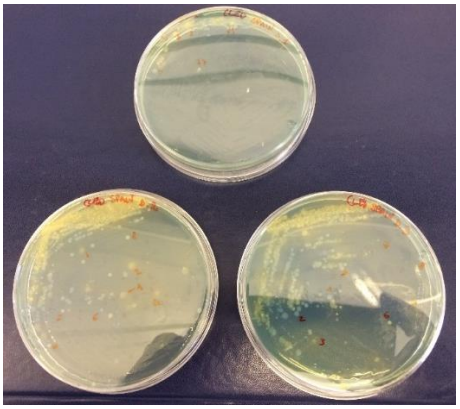


CASTELLON

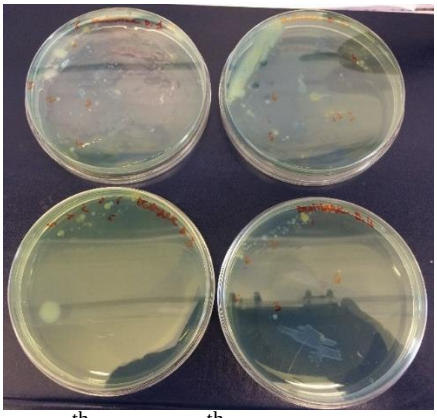
1st , 2nd and 3rd Day

LYNGBY

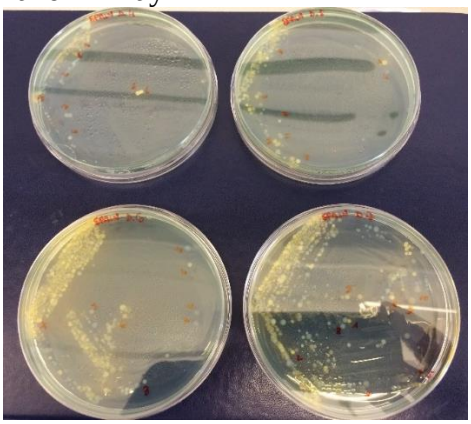
From 1st Day to 4th Day



5th and 7th Day

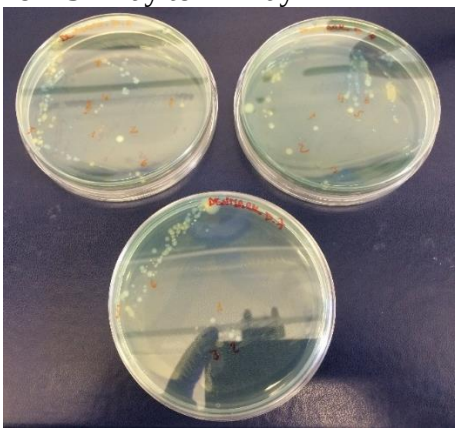


From 5th Day to 7th Day



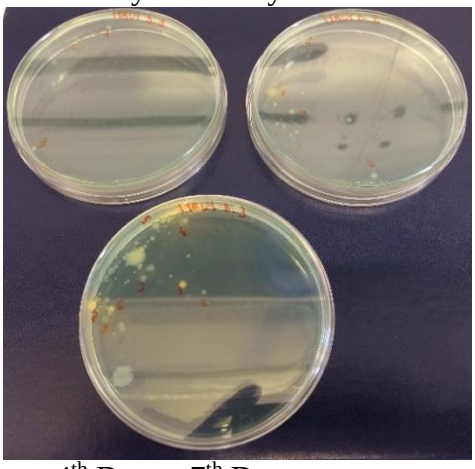
MILAN

From 1st Day to 3rd Day

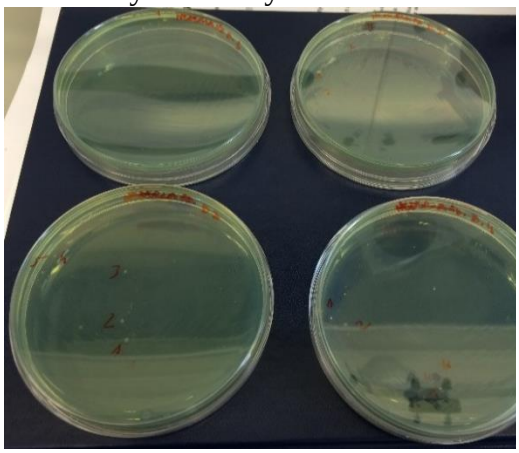


UTRECHT

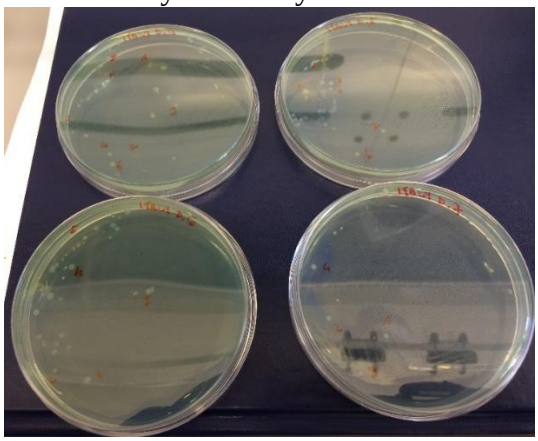
From 1st Day to 4th Day

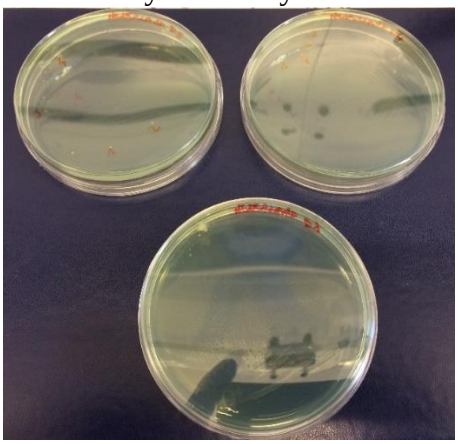


From 4th Day to 7th Day



From 5th Day to 7th Day





ZURICH

1st, 2nd, 6th and 7th Day

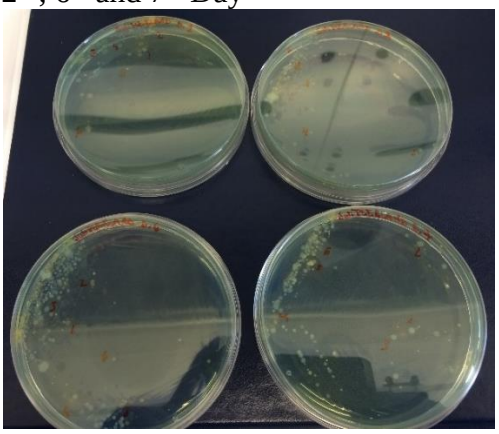


**Figure S4** Result of the incubation for the colonies found from every day of the sampling campaign in Oslo, Castellon, Lyngby, Milan, Utrecht and Zurich.

**Table S2** Results on colony morphology assessment for the selected cities in the study.

| **City** | **COLONY MORPHOLOGY ON CLED AGAR** | | | | |
|---|---|---|---|---|---|
| **OSLO** | **Light cream morphology** | **Dark cream morphology** | **Fuzzy dots** | **Golden colony** | **Pale yellowish** |
| *1st Day* | 1, 3 | 2, 4 | | | |
| *2nd Day* | 1, 4 | 2, 3 | | | |
| *3rd Day* | 3, 4 | 1, 2 | | | |
| *4th Day* | 2, 3 | 1, 4 | | | |
| *5th Day* | 4 | 1, 2, 3 | | | |
| *6th Day* | 3, 4 | 1, 2 | | | |
| *7th Day* | 2, 3 | 1, 4 | | | |
| **BRISTOL** | | | | | |
| *1st Day* | 1, 2, 3, 4, 5, 8, 10, 11 | 12, 13, 14, 15 | 6, 7 | 9, 16 | |
| *2nd Day* | 1, 2, 3, 11, 12, 13, 14, 15 | 4, 5, 6, 7, 8, 9, 10 | - | 16 | |
| *3rd Day* | 1, 3, 4, 5, 6, 10, 11, 12 | 2, 7, 8, 9, 13, 14 | 15, 16 | - | |
| *4th Day* | 1, 2, 3, 4, 5, 6, 7, 10, 11, 12, 13, 14 | 16 | 15 | 8, 9 | |
| *5th Day* | 1, 2, 3, 4, 14, 15, 16 | 6, 9, 10, 11, 12, 13 | - | 5, 7, 8 | |
| *6th Day* | 6, 7, 8, 12 | 1, 2, 3, 4, 5, 9, 10, 11, 13 | 14, 15, 16 | - | |
| *7th Day* | 11, 13, 14, 16 | 9, 10, 12 | 6, 7, 8 | 1, 2, 3, 4, 5, 15 | |
| **LYNGBY** | | | | | |
| *1st Day* | 2, 4 | 1, 3, 5 | - | - | 5 |
| *2nd Day* | 6, 4, 5 | - | - | 1, 2, 3 | - |
| *3rd Day* | 4, 5, 6 | 2, 3 | - | - | 1 |
| *4th Day* | 3, 5 | 4, 6 | - | 1, 2 | - |
| *5th Day* | 2, 3, 4 | 5, 6 | 1 | - | - |
| *6th Day* | 1, 6, 3 | 2 | - | 5, 4 | - |
| *7th Day* | 1, 2, 4 | 5, 6 | - | - | 3 |
| **UTRECHT** | | | | | |
| *1st Day* | - | - | - | - | - |
| *2nd Day* | 1, 2, 3, 4 | - | - | - | - |
| *3rd Day* | 1, 2, 3, 4, 5 | - | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| *4th Day* | 2, 4 | 1, 3 | - | - | - |
| *5th Day* | 1, 2, 3, 4 | - | - | - | - |
| *6th Day* | 1, 4 | 2, 3 | - | - | - |
| *7th Day* | - | - | - | - | - |
| **ZURICH** | | | | | |
| *1st Day* | 1, 2, 4, 5 | - | - | 3 | 6 |
| *2nd Day* | 3, 4, 5 | - | - | 1, 2 | 6 |
| *6th Day* | 1, 3, 6 | - | - | - | 2, 4, 5 |
| *7th Day* | 1, 2, 3 | 5 | - | - | 4, 6 |
| **MILAN** | | | | | |
| *1st Day* | 1, 2, 3 | - | - | - | - |
| *2nd Day* | 1, 2, 4, 5 | - | - | - | 3 |
| *3rd Day* | 2, 3 | 6, 7 | 4 | - | 1, 5, 8 |
| *4th Day* | 3, 4, 5, 6, 7, 8 | - | - | - | 1, 2 |
| *5th Day* | 2, 3, 4, 6 | 1 | - | - | 5 |
| *6th Day* | 1, 2, 3, 4, 5 | - | - | - | - |
| *7th Day* | 1, 2, 3, 4 | - | - | - | - |
| **CASTELLON** | | | | | |
| *1st Day* | 1, 2, 5, 6, 7 | 3 | - | 4, 8 | - |
| *2nd Day* | - | 5, 6, 7, 8 | - | - | 1, 2, 3, 4 |
| *3rd Day* | 1, 2 | 5, 6 | - | 7, 8 | 3, 4 |
| *4th Day* | 2, 3, 4, 8 | 5, 6 | - | 7 | 1 |
| *5th Day* | 4, 5, 7, 8 | 1, 2, 3, 6 | - | - | - |
| *6th Day* | 1, 2, 3, 5 | 4, 8 | - | - | 7, 6 |
| *7th Day* | 7, 8, 10 | 1, 2, 4 | 5, 6 | - | 3, 9 |

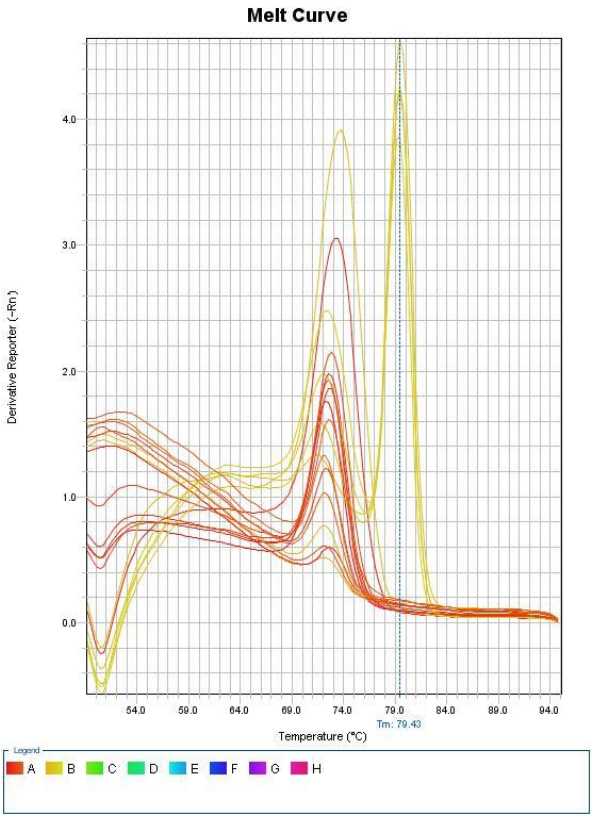



**Figure S5** qPCR-Melt curve.

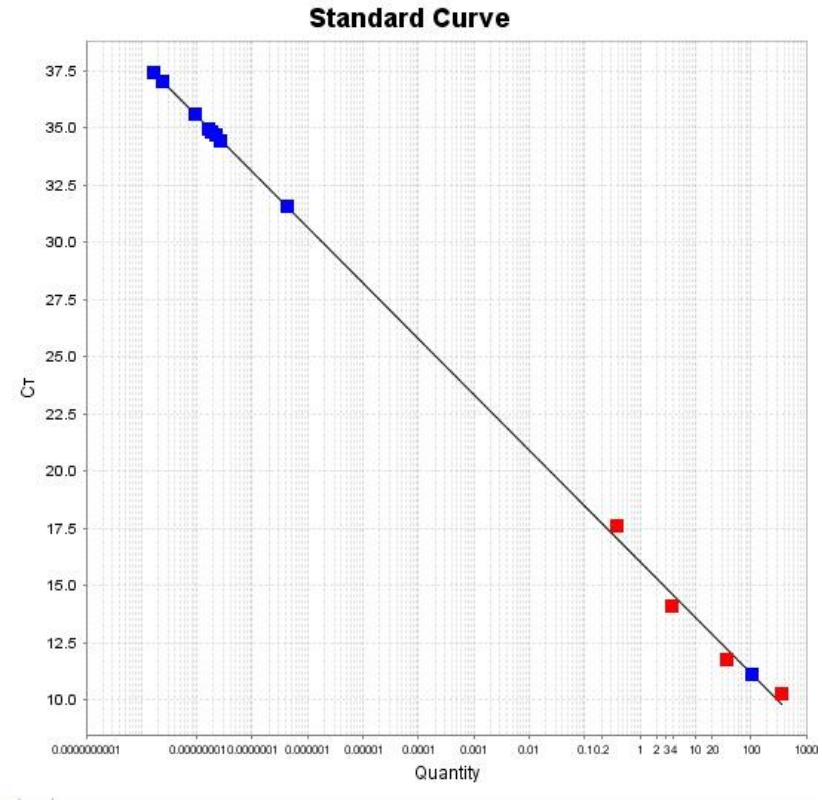



**Figure S6** qPCR-Standard curve.